MOLECULAR CHARACTERIZATION AND DETECTION OF DASHEEN MOSAIC VIRUS

By

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TABLE OF CONTENTS

LIST OF FIGURES

LIST OF TABLES

KEY TO ABBREVIATIONS

AI	amorphous inclusion
bp	base pair
BCMV	bean common mosaic virus
BlCMV	blackeye cowpea mosaic virus
BYMV	bean yellow mosaic virus
CP	coat protein
C-terminus	carboxy-terminus
cDNA	complementary DNA
CMV	cucumber mosaic virus
CI	cylindrical inclusion
µCi	microCurie
DIECA	diethyldithiocarbamate
DSMO	dimethyl sulfoxide
DsMV-Ce	taro isolate of dasheen mosaic virus
DsMV-Ch1	caladium isolate of dasheen mosaic virus from cultivar `Candidum'
DsMV-Ch2	caladium isolate of dasheen mosaic virus from cultivar `Carolyn Whorton'
DsMV-Ch3	caladium isolate of dasheen mosaic virus from cultivar `Frieda Hemple'
DsMV-Xc	cocoyam isolate of dasheen mosaic virus
DsMV-Za	calla lily isolate of dasheen mosaic virus
ELISA	enzyme-linked immunosorbent assay
HC/Pro	helper component/protease
IPTG	isopropyl-ß-D-thiogalactopyranoside
kb	kilobase
kDa	kilodalton
LB	Luria broth
β-ME	β-mercaptoethanol
MW	molecular weight
3'-NCR	3' non-coding region
N-terminus	amino-terminus
NIa	nuclear inclusion a
NIb	nuclear inclusion b
nm	nanometer
nt	nucleotide
oligo dT	oligonucleotide deoxythymidine

PCR	polymerase chain reaction
PepMoV	pepper mottle virus
PMoV	peanut mottle virus
PPV	plum pox virus
PRSV-W	papaya ringspot virus type W
PRSV-P	papaya ringspot virus type P
PSbMV	pea seed-borne mosaic virus
PStV	peanut stripe virus
PVY	potato virus Y
RT-PCR	reverse transcription-polymerase chain reaction
SbMV	soybean mosaic virus
SCMV	sugarcane mosaic virus
SDS-PAGE	sodium dodecyl sulfate polyacrylamide gel electrophoresis
TEV	tobacco etch virus
TuMV	turnip mosaic virus
TVMV	tobacco vein mottling virus
WMV 2	watermelon mosaic virus 2
X-Gal	5-bromo-4-chloro-3-infolyl-ß-D-galactopyranoside
ZYMV	zucchini yellow mosaic virus

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The sequence of the 3'-terminal 3158 nucleotides of a caladium (*Caladium hortulanum*) isolate of dasheen mosaic virus (DsMV-Ch1) was determined. The region contains the nucleotide sequence which encodes the carboxyl terminus of the NIa protease, the NIb RNA polymerase, and the coat protein (CP). The genomic organization of this region is similar to those of other potyviruses. The overall nucleotide sequence homology of the coding region compared with those of other sequenced potyviruses is between 57-67%, and the amino acid sequence homology is between 68-82%. Phylogenetic alignment of the genomic sequences indicated that DsMV is a distinct member of the genus *Potyvirus* in the family *Potyviridae*.

The CP gene of DsMV-Ch was amplified by PCR, cloned into a pETh-3 vector, and expressed in *Escherichia coli*. Antiserum against the expressed CP was obtained and it was suitable for detecting DsMV in SDS-diffusion test, ELISA and Western blotting.

Variability among DsMV isolates from caladium, calla lily (*Zantedeschia spp.*), cocoyam (*Xanthosoma spp.*), and taro (*Colocasia esculenta*) was noted in symptom severity in inoculated *Philodendron selloum* seedlings. The CP molecular weight (MWs) among isolates varied, ranging from 38-47 kDa in their original hosts based on western blotting analysis. Because respective MWs of each DsMV isolate remained constant after three passages in *Philodendron selloum* or other hosts, it was concluded that the observed differences in the CP MWs were virus-mediated. Comparison of the CP sequence among DsMV isolates revealed deletions and additions at the 3'-terminal regions, which may contribute to the variability of the CP MWs among different DsMV isolates.

DsMV was not uniformly distributed within tissues of infected cocoyam and taro leaves. In contrast, more uniform distribution of the virus was noted within infected `Candidum', `Carolyn Whorton', and `Frieda Hemple' caladium leaves. Relatively high growing temperatures resulted in a reduction in the distribution of DsMV within leaves of infected cocoyam and taro plants. Under such conditions, many leaves without detectable virus were produced by infected plants, which may be related to restriction of virus movement in these plants.

Immunosorbent electron microscopy (ISEM), ELISA, Western blotting, and reverse transcription-PCR (RT-PCR) were used to detect different DsMV isolates in leaves and/or corms of caladium, calla lily, cocoyam and taro. Of them, RT-PCR was the most sensitive. DsMV detection was also facilitated by corm wounding.

CHAPTER 1
INTRODUCTION

Dasheen mosaic virus (DsMV) is a species of the genus *Potyvirus* in the family *Potyviridae*, and infects plants in the family *Araceae*, collectively referred to as aroids. This potyvirus causes stunting and foliar mosaic, chlorotic feathering, and distortion symptoms in infected plants, thereby reducing their market values and/or yields (Zettler & Hartman, 1987).

General Characteristics of *Potyviridae*

The *Potyviridae* is the largest of 47 plant virus groups and families currently recognized by the International Committee for the Taxonomy of Viruses. Three genera, *Potyvirus*, *Rymovirus*, and *Bymovirus*, are recognized (Murphy et al., 1995). There are two whitefly transmitted viruses and two aphid transmitted viruses are unassigned. The family contains at least 184 definitive and possible species (30% of all known plant viruses), many of which cause significant losses in agronomic, pasture, horticultural and ornamental crops (Shukla et al., 1994). A feature shared by all potyviruses is that they induce characteristic cylindrical inclusion bodies in the cytoplasm of the infected cells (Edwardson, 1974). These cylindrical inclusion (CI) bodies are formed by a virus-encoded protein (Dougherty & Hiebert, 1980). Except for species of the genus *Bymovirus,* which have bipartite particles about 500-

600 nm and 250-300 nm long, *Potyvirus* and *Rymovirus* virions are flexuous filaments of 650 to 900 nm in length (Murphy et al., 1995). The particles of monopartite potyviruses contain one positive-sense, single-stranded genomic RNA molecule of 8.5-10 kb, which is encapsidated by a single type of coat protein (Hollings & Brunt, 1981). The genomic RNA has a protein (VPg) covalently attached to its 5' end (Hari, 1981; Siaw et al., 1985; Riechmann et al., 1989; Murphy et al., 1990) and a poly(A) tract at its 3' end (Hari, 1979). Most potyviruses (*Potyvirus*) are transmitted by aphids in a non-persistent manner, while *Rymovirus, Bymovirus,* are transmitted by mites, and fungi, respectively. Some potyviruses, such as TEV, are known to form nuclear inclusion bodies consisting of two nuclear proteins (NIa & NIb) aggregated in equimolar amounts in nuclei of infected cells. A few potyviruses, such as PRSV-W, PeMoV, potato virus A (PVA) and celery mosaic induce the formation of amorphous inclusions in the cytoplasm and/or in the nucleoplasm of infected cells (Christie & Edwardson, 1977; Edwardson & Christie, 1983).

Genome Organization

The complete nucleotide sequence of the following thirteen potyviruses has been documented: TEV (Allison et al., 1986), TVMV (Domier et al., 1986), PPV (Maiss et al., 1989; Lain et al., 1989), PepMoV (Vance et al., 1989), the necrotic and Hungarian strains of PVY (Robaglia et al., 1989, Thole et al., 1993), PSbMV (Johansen et al., 1991), PRSV-P (Yeh et al., 1992), the two strain of SbMV (Jayaram et al., 1992), TuMV (Nicolas et al., 1992), Johnsongrass mosaic (Gough & Shukla, 1993), PVA (Puurand et al., 1994), PStV

(Gunasinghe et al., 1994) and ZYMV (Wisler et al., 1995). The sequence analysis of potyviruses and *in vitro* translation studies of potyvirus genomic RNAs have revealed a single open reading frame (ORF) encoding a large polyprotein ranging from 320 kDa to 358 kDa, depending on the virus. This polyprotein is proteolytically processed into at least eight mature viral proteins by three virus-encoded proteases (Hellmann et al., 1983; Dougherty & Carrington, 1988). The different gene products into which the potyviral polyprotein is cleaved are, proceeding from the N- to the C-terminus of the polyprotein: P1 protease, the helper component/protease protein (HC-Pro), P3, a putative 6K peptide (6K1), the CI protein with helicase activity, a second 6K peptide (6K2), the nuclear inclusion "a" protein (NIa), which functions as VPg and protease, the nuclear inclusion "b" protein (NIb), the presumptive RNA polymerase, and the capsid protein (CP). The length of potyviral 5' non-coding regions ranges from 85 nucleotides (nt) for PRSV-W to 205 nt for TVMV. These regions are especially rich in adenine residues with relatively few guanine residues. It has been shown that the TEV 5' non-coding region can function as an enhancer of translation (Carrington et al., 1990). Alignment of the non-coding regions of PPV, PVY, TEV, and TVMV revealed two highly conserved regions, namely box "a" (ACAACAU) and box "b" (UCAAGCA) (Lain et al., 1989; Turpen, 1989). These conserved sequences and their secondary structure may be important for processes such as encapsidation, translation or replication (Lain et al., 1989; Atreya et al., 1992; Riechmann et al., 1992).

The 3' non-coding regions of different potyviruses have been described as variable in size, sequence, and predicted secondary structure (Lain et al., 1988; Turpen, 1989; Quemada

et al., 1990a, b). They contain AU-rich segments, and each sequence can be predicted to fold into stable secondary structures (Turpen, 1989). Several short segments displaying sequence homology among different potyviruses have been identified (Lain, 1989; Uyeda et al., 1992). In contrast with the high sequence diversity found among the 3' non-coding regions of different potyviruses, the 3' non-coding regions are more conserved among different strains of the same potyvirus (Wetzel., 1991). The poly(A) tails have been determined to be very variable in length (Allison et al., 1986; Lain et al., 1988). The most important functions of the 3' non-coding region involve the interaction with virus replicase during the initiation of minus-strand RNA synthesis and the prevention of exonucleolytic degradation (Bryan et al., 1992; Dolja & Carrington, 1992). It has been shown that the 3' non-coding region of TVMV can have a direct effect on the induction of disease symptoms (Rodriguez-Cerezo et al., 1991).

Replication

The subcellular site(s) of potyviral RNA synthesis has not been identified with certainty, but is believed to be in the cytoplasm, as found with other positive stranded RNA viruses (Verchot et al., 1991). A polymerase activity is associated with an enzyme complex isolated from plants infected with PPV (Martin & Garcia, 1991). Several viral proteins, including NIb, CI, VPg/NIa, and two small peptides (6K1 and 6K2), are believed to be involved in the replication process of potyviruses. The large nuclear inclusion protein, NIb, is the most conserved gene product of potyviruses and is believed to be the RNA-dependent RNA polymerase (RdRp) based on the presence of conserved sequence motif (GDD)

characteristic of these enzymes (Domier et al., 1987; Lain et al., 1989; Robaglia et al., 1989; Poch et al., 1989; Riechmann et al., 1992).

The CI protein of PPV has been shown to have nucleic acid-stimulated ATPase activity and to be able to unwind RNA duplexes (Lain et al., 1990, 1991). The PPV CI was able to unwind only dsRNA substrates with the 3' single-strand overhangs, indicating that the helicase activity functions from the 3' to the 5' direction (Lain et al., 1990). The CI proteins of potyviruses were found to contain a conserved nucleotide binding consensus sequence motif (GXXGXGKS) at the C-terminal region and were implicated as membrane-binding components of the replication complex (Domier et al., 1987).

In addition to the role of NIa in the proteolytic processing of the potyviral polyprotein, for which only its carboxyl half is required, its N-terminal part has been shown to be the VPg. The VPg of TVMV (Siaw et al., 1985) and PPV (Riechmann et al., 1989) have been identified as proteins of 24 kDa and 22 kDa, respectively. The TVMV VPg cistron has been mapped showing that the VPg is the N-terminal portion of the NIa protein (Shahabuddin et al., 1988). Likewise, the TEV VPg has been found to be either the 49 kDa NIa or its N-terminal 24 kDa half (Murphy et al., 1990). The VPg is attached to the 5' end of the RNA by means of a phosphate ester linkage to Y residues of the protein (Murphy et al., 1991). By analogy with other viral systems, VPg is believed to serve as the primer for viral RNA synthesis (Shahabuddin et al., 1988).

The NIa protease also may be involved in regulation of potyvirus replication. One level of control has been proposed to be the regulation of the expression of gene products by

sequential proteolytic events (Dougherty et al., 1989a, b). Another proposed level of control is that the subcellular localization of the NIa/NIb may play a regulatory role (Carrington et al., 1991).

Proteolytic Processing of Polyprotein

Three virus-encoded proteases, NIa (Carrington & Dougherty, 1987a; Hellmann et al., 1988; Chang et al., 1988; Garcia et al., 1989a; Ghabrial et al., 1990), HC-Pro (Carrington et al., 1989a), and P1 (Carrington et al., 1990; Verchot et al., 1991), process the large viral precursor polyprotein co- and post-transcriptionally. The NIa is responsible for cleavages in the C-terminal two-thirds of the polyprotein (Dougherty et al., 1988), whereas HC-Pro and P1 autocatalytically cleave at their respective C-termini (Carrington et al., 1989 a, b; Verchot et al., 1991).

The small nuclear inclusion protein, NIa, is the major protease of potyviruses, and it is capable of cleaving in a cis- and trans-manner at least six and possibly seven sites within the polyprotein. It has a two-domain structure where the N-terminal domain is the genome-linked VPg (Shahabuddin et al, 1988; Murphy et al., 1990), and the C-terminal half is the true proteinase (Dougherty & Carrington, 1988). This proteolytic domain in TEV and TVMV was like that reported for the poliovirus 3C and cowpea mosaic virus 24 kDa proteases (Domier et al., 1987). The NIa protease is related to the trypsin-like family of cellular serine proteases, except that a Cys is substituted for the active site nucleophile (Bazan and Fletterick, 1988; Gorbalenya et al., 1989). Mutagenesis of selected TEV 27-kDa NIa ORF codons supports the

hypothesis that His-46, Asp-81, and Cys-151 make up the active-site triad (Dougherty et al., 1989b). The consensus sequence GXCG has been found in all potyviruses examined to date (Shukla et al., 1994). The NIa autocatalytically releases from the polyprotein by cleaving the CI-NIa, and NIa-NIb junctions and catalyzes the production of CI, NIb, and CP by cleaving the P3-CI, and NIb-CP junctions (Carrington & Dougherty, 1987a, b; Carrington et al., 1988; Hellman et al., 1988; Garcia et al., 1989a, b, 1990). Additional cleavages, to release VPg and the 6K1 and 6K2 products, also occur (Garcia et al., 1992; Restrepo-Hartwig & Carrington, 1992).

The NIa protease requires those conserved cleavage sites, defined as heptapeptide sequences, that are efficiently recognized only by their own respective proteases (Carrington & Dougherty, 1987a, 1988; Carrington et al., 1988; Dougherty et al., 1988, 1989a; Garcia et al., 1989 a, b; Garcia & Lain, 1991; Parks & Dougherty, 1991). Cleavages are frequently at a Q/(G, or S) site. A group-specific motif VXXQ/(A, S, G, or V), common to most potyviruses, has been found (Shukla et al., 1994). The requirement for the conserved cleavage sites is unique to the NIa proteases.

The helper component protease (HC-Pro) functions as an autocatalytic protease. The HC-Pro 52 kDa protein of TEV is a multifunctional protein, and the proteolytically active domain has been localized at its C-terminal half (Carrington et al., 1989a, b). The presence of two essential residues, specifically Cys-679 and His-772, in this protease supports the hypothesis that HC-Pro most closely resembles members of the cysteine-type family of proteases (Oh & Carrington, 1989). Cleavage is at a specific G/G dipeptide and appears to be

the only cleavage event mediated by the HC-Pro protein (Carrington et al., 1989b; Oh & Carrington, 1989). The HC-Pro of potyviruses accumulates to high levels and often complexes into amorphous inclusion bodies (de Mejia et al., 1985b)

By expressing TEV polyprotein in transgenic plants, it was shown that a novel proteolytic activity caused by neither HC nor NIa proteases is required for processing at the C-terminal region of P1 protein (Carrington et al., 1990). Verchot et al. (1991) have demonstrated that P1 is the protease responsible for cleaving the P1-HC junction at the Q/(S, or G). Using the wheat germ *in vitro* translation system and a series of truncated or mutagenized cDNAs from TEV, they showed that most of the HC protein and the first 157 amino acids of P1 were not required for proteolysis of the P1-HC junction and that the N-terminal boundary of the protease domain lies somewhere between 157-188 and 304. The P1 protease is a serine-like protease based on the presence of the conserved active-site triad (His-215, Asp-225 and Ser-256 for TEV) and the conserved motif (GXSG) found in all aphid-transmitted potyviruses (Lain, 1990; Verchot, 1991). However, another factor besides P1 might be required since cleavage at this site does not occur in an *in vitro* rabbit reticulocyte lysate system (Hiebert et al., 1984b; Carrington et al., 1989a). Alternatively, the absence of cleavage in the reticulocyte lysate-based system could be due to the presence of a protease inhibitor (Verchot et al., 1991).

Virus Movement

Natural plant-to-plant spread of the majority of potyviruses is accomplished by aphids, and four viral proteins, P1, HC/Pro, CI and CP, have been suggested or demonstrated to be involved in either cell-to-cell movement or plant-to-plant spread. Based on the sequence similarity of the P1 protein of TVMV to that of 30 kDa movement protein of tobacco mosaic virus, it has been suggested that the P1 protein may be involved in cell-to-cell movement (Domier et al.,1987; Lain et al., 1989a; Robaglia et al., 1989). However, sequence identity may be a poor indicator of function since it is known that cell-to-cell movement proteins of plant viruses exhibit very little similarity, even among members of the same group (Lain et al., 1989a; Hull, 1991). The P1 protein of other potyviruses (TEV, PPV, PVY), for example, differed from that of TVMV. The P1 proteins of the potyviruses are the most variable products of the genome (Wisler et al., 1995), which suggests that P1, particularly its N-terminal non-proteases domain, may be involved in some specific virus-host interaction (Hull, 1991). By deletions and modifications of the P1 coding sequence, Verchot and Carrington recently (1995) demonstrated that P1 protein of TEV was not involved in the movement.

The HC protein is involved in aphid transmission and must be acquired by the insect in conjunction with the virus (Pirone & Thornbury, 1983; Thornbury & Pirone, 1983; Hiebert et al., 1984; Thornbury et al., 1985; Berger & Pirone, 1986). Although the HC protein is closely related to the protein associated with the amorphous inclusions induced by certain potyviruses (Hiebert et al., 1984; De Mejia et al., 1985 a, b; Baunoch et al., 1990), functional studies have

suggested that either the inclusion-bound form of this protein has been inactivated or, alternatively, the HC activity is associated with a modified form of the inclusion protein (Thornbury & Pirone, 1983; Thornbury et al., 1985; Dougherty & Carrington, 1988). The size of the biologically active HC form is believed to be a dimer, with MWs of 116 kDa for PVY (Hellmann et al., 1983) and 106 kDa for TVMV (Thornbury et al., 1985). The loss of transmissibility associated with HC deficiency has been correlated with two mutations in the HC coding sequence of potato virus C (Thornbury et al., 1990; Atreya et al., 1992) and the PAT isolate of ZYMV-PAT (Granier et al., 1993). The long-distance movement of TEV has been associated with the central region of the HC-Pro by using site-directed mutagenesis of infectious cDNA and complementary by HC-Pro supplied in *trans* by a transgenic host (Cronin et al., 1995).

The CI protein has also been suggested to be involved in cell-to-cell movement on the basis of electron microscope observations that CIs are associated with plasmodesmata and virus particles (Lawson & Hearon, 1971; Murant et al., 1971; Langenberg, 1986; Lesemann, 1989; Baunoch et al., 1991).

The coat protein is the most extensively characterized potyviral gene product. The CP nucleotide sequences of 103 strains of 35 distinct members of the *Potyviridae* have been resolved (Shukla et al., 1994; Pappu et al., 1994; Puurand et al., 1994; Husted et al., 1994; Colinet & Lepoivre, 1994). The interest in CP comes mainly from its usefulness in taxonomic and evolution studies, in diagnosis, and in the study of CP-mediated resistance. Sequence comparisons and particle assembly properties suggest the presence of three different regions in

the coat protein molecules of potyviruses: (i) a surface-exposed N-terminus varying in length and sequence, (ii) a highly conserved core of 215-227 amino acids, and (iii) a surface-exposed C-terminus of 18-20 amino acids (Shukla and Ward, 1989). Removal of the N- and C-termini by trypsin digestion leaves a fully assembled virus particle composed of the coat protein core region, which can not be distinguished by electron microscopy from untreated native infective particles. Apparently the N- and C-termini are neither required for particle assembly nor for infectivity during mechanical inoculation (Shukla et al., 1988; Jagadish et al., 1991).

The CP functions to protect the viral RNA, to facilitate its transmission by aphids (Gal-On et al., 1990; Lecoq & Purcifull, 1992), and to facilitate movement of the virus within plants (Dolja et al., 1994, 1995). Sequence analyses have shown that a change in the amino acid triplet DAG, which is conserved in all aphid-transmissible potyviruses (Harrison & Robinson, 1988; Atreya et al., 1991), and other amino acids in the amino-terminus (N-terminus) of the CP alters aphid transmissibility (Atreya et al., 1990, 1991, 1995; Harrison & Robinson, 1988; Gal-On et al., 1990; Salomon & Raccah, 1990). A non-aphid transmissible isolate of ZYMV, which has a defective CP but is capable of producing an active form of HC, has been described (Antignus et al., 1989; Gal-On et al., 1992). By using mutational analysis, Atreya et al. (1995) demonstrated that a basic residue (D or N) in the first position, the nonpolar residue A in the second position, and the small nonpolar residue G in the third position are required for aphid transmissibility.

The TEV CP has been recently shown to be necessary for cell-to-cell movement and long-distance transport of the virus in plants (Dolja et al., 1994, 1995). The mutation at the

highly conserved S amino acid residue in the core domain and deletion at the variable C-terminal region abolished or reduced virus movement within the plants.

In addition to their natural functions, the CP genes of some potyviruses, including SbMV (Stark & Beachy, 1989), PPV (Reger et al., 1989; Scorza et al), PVY (Kaniewski et al., 1990), PRSV-P (Ling et al., 1991; Fitch et al., 1992), TEV (Lindbo & Dougherty, 1992), WMV-2 (Namba et al., 1992), ZYMV (Namba et al., 1992; Fang & Grumet, 1993), TVMV (Zaccomer et al., 1993), and lettuce mosaic virus (Dianat et al., 1993) have been used experimentally to obtain genetically engineered plants with CP-mediated resistance. Many of these transgenic plants showed certain degrees of resistance to viral infection.

Dasheen Mosaic Virus

Dasheen mosaic virus (DsMV) is a species of the *Potyviridae* which causes serious diseases of cultivated aroid plants worldwide (Zettler et al., 1978; Shimoyama et al., 1992a, b). Viruses other than DsMV include konjak mosaic potyvirus of *Amorphophallus* (Shimoyama et al., 1992a, b); tobacco necrosis necrovirus of *Dieffenbachia* (Paludan & Begtrup, 1982); cucumber mosaic cucumovirus of *Arum* (Lovisolo & Conti, 1969), *Amorphophallus* (Shimoyama et al., 1990) and *Colocasia* (Kumuro & Asuyama, 1955); tomato spotted wilt tospovirus of *Zantedeschia* (Tompkins & Severin, 1950); and bobone rhabdovirus of *Colocasia* (James et al., 1973). DsMV and konjak mosaic virus are considered different on the basis of biological and serological properties. However, none of these viruses infect as many aroids nor is as wide spread as DsMV.

The *Araceae*, or aroid family, comprises about 107 genera and 2,500 species of monocotyledonous herbs and vines. Most aroid plants occur in tropical Asia and the New World tropics (Grayum, 1990). Many of them, such as *Aglaonema, Arisaema, Caladium, Dieffenbachia, Epipremnum, Monstera, Philodendron, Pinellia, Spathiphyllum*, and *Syngonium* are important ornamentals, which account for nearly 25% of U.S. production of foliage plants (U.S. Bureau of Census, 1974). Certain species of *Anthurium, Richardia*, and *Zantedeschia* are valuable cut flower crops, and *Cryptocoryne* species are commercially grown aquarium plants. Two genera of aroids, *Colocasia,* commonly referred as dasheen or taro, and *Xanthosoma*, or cocoyam, are important tropical food crops. DsMV was first reported in 1970 in Florida by Zettler et al. (1970), and has since been found elsewhere, including Hawaii (Buddenhagen et al, 1970; Hartman & Zettler, 1972; Kositratana et al, 1983), Puerto Rico (Alconero & Zettler, 1971), Trinidad (Kenten & Woods, 1973), India (Hartman, 1974), Venezuela (Debrot & Ordosgoitti, 1974), Japan (Tooyama, 1975), Egypt (Abo-Nil & Zettler, 1976), Netherlands (Hakkaart & Waterreus, 1976), the Solomon Islands (Gollifer et al. 1977), Belgium (Samyn & Walvaert, 1977), Papua New Guinea (Shaw et al., 1979), Great Britain (Hill & Wright, 1980), the Cameroons (Girard et al., 1980), Kiribati (Shanmuganathan, 1980), French Polynesia (Jackson, 1982), Nigeria (Volin et al., 1981), Italy (Rana et al., 1983), South Africa (Van der Meer, 1985), Costa Rica (Ramirez, 1985), Australia (Greber & Shaw, 1986), P. R. China (Zettler et al., 1987), Taiwan (Ko et al., 1988) and Cuba (Quintero, 1989). Although DsMV has been reported to experimentally infect nonaroids such as *Chenopodium amaranticolor, C. quinoa, C. ambrosioides, Nicotiana benthamiana* and *Tetragonia expansa*

(Gollifer & Brown, 1972; Rana et al., 1983; Kositratana, 1985; Shimoyama et al., 1992a), its natural host range is restricted to aroid plants, and it has been reported to infect species of 20 genera: *Aglaonema, Alocasia, Amorphophallus, Anthurium, Arisaema, Caladium, Colocasia, Cryptocoryne, Cyrtosperma, Dieffenbachia, Monstera, Philodendron, Pinellia, Richardia, Scindapsus, Spathiphyllum, Stenospermation, Syngonium, Xanthosoma* and *Zantedeschia* (Zettler et al., 1987; Samyn & Welvaert, 1977; Chen, personal communication).

As noted for other *Potyviridae*, DsMV has flexuous, filamentous particles about 750 nm long (Zettler et al., 1978; Samyn & Welvaert, 1977; Hill & Wright, 1980; Girard et al., 1980; Kositratana et al, 1983; Van der Meer, 1985; Greber & Shaw, 1986; Quintero, 1989). It induces cylindrical inclusions in infected cells (Zettler et al., 1978; Girard et al, 1980; Shanmuganathan,1980; Paludan & Begtrup, 1982; Greber & Shaw, 1986; Kositratana, 1985; Ko et al., 1988, Liang et al., 1994), and like other members of this genus, DsMV is sap-transmissible. DsMV also is transmitted in a non-persistent manner by aphids, namely *Myzus persicae*, *Aphis craccivora* (Morales & Zettler, 1977; Van der Meer, 1985), and *Aphis gossypii* (Gollifer et al, 1977), but apparently not by either *Pentalonia nigronervosa* (Morales & Zettler, 1977) or *Rhopalosiphum padi* (Gollifer et al., 1977).

The genome of DsMV is a single-stranded RNA of MW 3.2-3.42 x 10^6 (Kositratana, 1985; Shimoyama et al., 1992b). The four nonstructural proteins that have been identified thus far were HC-Pro (51 kDa), CI (69 kDa), NIa (49 kDa), and NIb (56 kDa) proteins (Nagel & Hiebert, unpublished). The DsMV CP protein is serologically related to those of araujia mosaic, BlCMV, TEV and ZYMV (Abo El-Nil et al., 1977; Hiebert & Charudattan, 1984;

Kositratana, 1985). The DsMV CI protein is serologically related to that of araujia mosaic virus (Hiebert & Charudattan, 1984), and the DsMV *in vitro* synthesized protein is related to the TVMV HC-Pro protein (Hiebert et al., 1984). The 3'-terminal region and the CP gene of two *Colocasia* isolates of DsMV from Florida have been cloned and sequenced (Pappu et al., 1993, 1994a, b). The predicted CP of isolate DsMV-LA contains 329 amino acids and has an estimated MW of 36.2 kDa, and the CP of isolate DsMV-TEN contains 314 amino acids and has a MW of 34.6 kDa. The CP sequence comparisons and phylogenetic reconstructions indicated that the DsMV is a distinct potyvirus within the passionfruit woodiness virus subgroup cluster.

Symptoms caused by DsMV in nature may differ considerably according to the aroid host infected and the season in which the host is grown. In some aroids such as *Colocasia, Richardia, Xanthosoma, Zantedeschia* and certain *Dieffenbachia* cultivars, DsMV causes leaf mosaic, leaf mottle, chlorotic streaking along veins on leaves, and leaf distortion. The inflorescence of *Zantedeschia* may show color break, with blisters and malformation (Zettler et al., 1970; Alconero & Zettler, 1971; Hakkaart & Waterreus, 1976; Hill & Wright, 1980; Paludan & Begtrup, 1982; Van der Meer, 1985; Greber & Shaw, 1986). In other aroids, such as *Aglaonema* and *Spathiphyllum*, DsMV symptoms are usually much less evident. A characteristic of many aroids is that DsMV symptoms are intermittently expressed, often making detection difficult. In some instances, such as with *Colocasia, Dieffenbachia, Richardia*, symptom expression is seasonal, most often appearing on foliage produced during fall and/or spring months (Chase & Zettler, 1982; Greber & Shaw; 1986). Some aroid cultivars

more readily express DsMV symptoms than others. The caladium cultivars, `Candidum' and `White Christmas', for example, are much more likely to exhibit symptoms throughout the growing season than the cultivars `Frieda Hemple' and `Carolyn Whorton' (Zettler & Hartman, 1986). The virus can cause yield loss of up to 60% in *Caladium*, *Dieffenbachia*, *Philodendron* and *Zantedeschia* (Zettler & Hartman, 1987).

Diagnosis and detection of DsMV have been based on techniques of bioassay, serology, and/or light and electron microscopy. *Philodendron selloum* seedlings are very susceptible to infection of DsMV and have been used frequently in bioassays (Zettler et al., 1970; Paludan & Begtrup, 1982); however, the seed viability of this and other aroids is short, and the seed are not readily available commercially (Zettler & Hartman, 1987). Since DsMV is not the only potyvirus which infects aroids (Shimoyama et al., 1992a; Chen, personal communication), light and electron microscopy can not necessarily be used as reliable evidence for ascertaining the existence of DsMV (Zettler & Hartman, 1987), nor are these methods likely to be as sensitive as some others (Greber & Shaw, 1986). Serological methods, such as immunodiffusion tests, have been used extensively in diagnosis and detection of DsMV, but this method requires larger quantities of antiserum than techniques such as ELISA (Zettler & Hartman, 1986). ELISA was also reported to be used for DsMV detection (Rana et al., 1986 ; Hu et al., 1995), but either the antiserum used reacted with host proteins or no difference could be detected among different isolates.

Dasheen mosaic virus has been successfully controlled by tissue culture methods in some greenhouse grown aroids such as *Anthurium*, *Dieffenbachia*, *Philodendron*,

Spathiphyllum, Syngonium and *Zantedeschia*, although the primary purpose of the tissue culture method for these plants is rapid *in vitro* propagation (Zettler & Hartman, 1986, Gomez et al., 1989). Despite these techniques, DsMV still causes problems in some low-cash field-grown aroids such as *Caladium, Colocasia* and *Xanthosoma*, (Zettler et al. 1991).

Dasheen mosaic occurs throughout the world, due to the international distribution of aroids as food plants and ornamentals and the perpetuation of the virus by propagating plants vegetatively. Little is known about the evolutionary relationships among DsMV isolates occurring in different geographic areas and among various hosts. There is evidence for the occurrence of a severe strain of the virus in Frech Polynesia (Jackson, 1982). Symptomatic and serological differences were noticed between an Egyptian isolate and a Florida isolate of DsMV from taro (Abo El-Nil et al., 1976). Symptomatic and serological differences between a Fiji isolate and a Florida isolate from taro were also noted (Abo El-Nil., 1977). Differences in growth rate between *P. selloum* seedlings inoculated with several DsMV isolates were reported, in which 79.5% and 4% weight reduction for taro and dieffenbachia isolates, respectively, were reported (Wisler et al., 1978). The DsMV antiserum against a Chinese evergreen isolate reacted with homologous isolate but not Florida and Fuji isolates in DAS-ELISA (Kositratana, 198?). Shimoyama et al. (1992a) reported that the DsMV antiserum they prepared did not react with several other potyviruses, including PVY-T, WMV-2, ZYMV, BYMV, TuMV, BlCMV, SbMV and konjak mosaic viruses. These studies indicated that distinct DsMV isolates do exist, although the relationships between them remain obscure.

Other studies, in contrast, such as that by Zettler et al. (1987) indicate only slight differences among DsMV isolates.

As an important group of ornamentals, domestic and international movement of aroids occurs on a large scale. In addition to commercial bulk shipments of plant materials, there is considerable movement of small quantities of clonal plant germplasm for purposes of establishing botanical collections, breeding programs and medicinal plants. In order to avoid the spread of some aroid diseases, including DsMV, international guidelines for the safe movement of aroid germplasm have been recommended (Zettler et al., 1989). These guidelines recommend growing plants in greenhouses and indexing them periodically for at least one crop cycle before certifying them as being virus-free. Such conditions, if implemented, would impose severe constraints in the international trade of these plants. Reliable, sensitive, practical, and rapid means for detecting DsMV could help overcome such problems. A better knowledge of the characteristics of DsMV, regarding both its general and molecular properties, would provide the basis needed for improving the detection of DsMV and for understanding the relationships among different DsMV isolates. The purposes of this study were to (i) purify the virus for molecular studies of DsMV, (ii) obtain and evaluate antiserum to be used to diagnose this virus. (iii) develop reliable and effective methods for detecting DsMV in propagating units of *Caladium, Colocasia* and *Xanthosoma.*

An isolate from caladium was purified and its viral RNA used to establish a cDNA library. The 3-terminal region of this isolate was sequenced and compared to those of other potyviruses. The CP gene was expressed in *Escherichia coli* and used to obtain the antiserum

that was useful for serological tests. It was determined that the CP of DsMV isolates varied in size and in nucleotide sequence. Serological procedures such as ELISA and Western blotting were developed for detecting DsMV. Also reported was the use of RT-PCR for DsMV detection.

CHAPTER 2
CLONING, SEQUENCING OF THE 3'-TERMINAL REGION AND EXPRESSION OF THE COAT PROTEIN OF DSMV-Ch1

Introduction

As a potyvirus, dasheen mosaic virus (DsMV) shares many properties with other aphid-borne potyviruses, such as having flexuous, filamentous particles, inducing formation of cylindrical inclusions (CI) in infected cells, having a positive-sense, single-stranded RNA genome, being sap- and aphid-transmissible, having a relatively restricted host range, and being serologically related to many other potyviruses (Zettler et al., 1978; Li et al., 1992). The RNA of a Florida DsMV isolate from taro (*Colocasia esculenta*) has been translated in the rabbit reticulocyte lysate *in vitro* system (Nagel & Hiebert, unpublished) to give five major polypeptides, namely the HC-Pro, CI, NIa, NIb and CP proteins. The molecular weight of the genomic RNA was estimated to be 3.2×10^6 for a California isolate from Chinese evergreen (Kositratana, 1985), and 3.42×10^6 for a Japanese DsMV isolate from taro (Shimoyama et al., 1992). The coat protein (CP) and the 3' non-coding region (3'-NCR) of two DsMV taro isolates, LA and TEN, have been sequenced recently (Pappu et al., 1994b). However, the characteristics of the DsMV genome organization and the sequences of other genes have not been studied. Furthermore, understanding the molecular characteristics of DsMV will help to define the virus and improve prospects fot its diagnosis and detection for the purposes of establishing practical quarantine regulations and facilitating the production of virus-free plants

through micropropagation. The only source of DsMV antiserum was that provided by Abo El-Nil et al. (1977), but this supply is nearly exhausted. DsMV isolates also cross reacted with PTY 1 monoclonal antibody, which is commercially available through Agdia Inc. (Elkhart, IN). However, since this and other monoclonal antisera may not react with some potyviruses or some potyviral isolates thereof (Jordan, 1992), there is an inherent risk of not detecting certain viruses or viral isolates lacking the epitopes common to most other potyviruses. In this study, the 3'-terminal region 3158 nucleotides of a DsMV caladium isolate was cloned, sequenced and analyzed. The DsMV CP was expressed in *Escherichia coli* and used to produce polyclonal antiserum for DsMV diagnosis and detection.

Materials and Methods

Virus Isolates

A `Candidum' caladium plant infected with an isolate of DsMV was maintained in a greenhouse and used to inoculate *P. selloum* seedling plants at the 7-8 leaf stage with an artist's airbrush gun. Source tissue was triturated in a mortar and pestle with 0.05 M potassium phosphate buffer (w/v, 1:20), pH 7.2, containing 600 mesh carborundum. The supernatant was added to a glass bottle, which was connected with a portable carbon dioxide tank, and was propelled onto the first two newly expanded leaves of *P. selloum* seedlings using carbon dioxide at a pressure of 40 pounds per square inch (Gooding & Ross, 1970).

Virus Purification

The ultracentrifugation method used for DsMV purification was similar to that for ZYMV described by Wisler (1992) with modifications. The leaf or root tissues of infected caladium, calla lily, cocoyam, taro or *Philodendron selloum* seedlings were homogenized in a cooled Waring blender for 1 min with 3 volumes of 0.3 M potassium phosphate buffer, pH 8.2, to which 0.6% sodium diethyldithiocarbamate (DIECA) and 0.2% β-mercaptoethanol (β-ME) were added. The homogenate was emulsified with 1:1 (w/v) cold trichlorofluorethane (Freon) for 30 sec. Following centrifugation at 2,500 *g* with a Sorvall high speed centrifuge (Du Pont Co., Wilmington, DE) for 10 min, the aqueous phase was filtered through four layers of cheese cloth. The suspension was centrifuged at 7,500 *g* for 10 min. Triton X-100 was added to the aqueous phase to a final concentration of 1%. The mixture was stirred for 20 min at 4°C. The mixture was then centrifuged in a Beckman Ti 70 rotor (Beckman Instruments, Inc., Palo Alto, CA) at 100,000 *g* (37k rpm) for 90 min. The pellet was resuspended in 20 mM HEPES, pH 8.2, containing 10 mM EDTA and 0.1% β-ME, with a tissue homogenizer. After stirring for one hour at 4°C, the suspension was partially clarified by centrifugation at 2,000 *g* for 10 min. The supernatant was layered on the top of Beckman SW41 tubes containing a Cs_2SO_4 solution (11.6 g salt plus 27 ml of 20 mM HEPES, pH 8.2), 5 ml per tube. The tubes were centrifuged at 140,000 *g* (32k rpm) for 16-18 hr at 4°C. The two opalescent virus zones 24-26 mm from the bottom of the tube were collected by droplet fractionation. The collected fractions containing the virions were combined and diluted with 1 volume of 20 mM HEPES, pH 8.2, and centrifuged at 10,000 *g* for 10 min. The virus was precipitated from the supernatant by

adding polyethylene glycol (PEG, MW 8,000) to a concentration of 6% (w/v), stirring at 4°C for 30 min, and then centrifuging at 10,000 *g* as before. The pellet was resuspended in 0.5 ml of 20 mM HEPES, pH 8.2. Concentrations of the virus preparations were estimated by spectrophotometry using an approximate extinction coefficient of A_{260} 2.6 (1 mg/ml, 1 cm light path). Five µl of a 1:20 dilution of the virus preparation was mixed with an equal volume of Laemmli dissociating solution (Laemmli, 1970), boiled for 2 min and analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). Gels were stained with Coomassie Brilliant Blue R-250 (Gibco BRL, Gaithersburg, MD), and destained in a solution containing 1% acetic acid and 10% methanol for visualization. The virus preparations were stored at -80°C, and then used for RNA isolation.

Viral RNA Isolation

Viral RNA was isolated from purified virus preparations by two methods, sucrose gradient centrifugation and phenol/ chloroform extraction.

A purified virus preparation (5 mg) was dissociated by incubation in an equal volume of RNA dissociating solution (0.2 M Tris-HCl, pH 9.0, containing 2 mM EDTA, and 2% SDS) and 400 µg/ml of protease K for 10 min at room temperature. Linear-log sucrose density gradients were made as described (Brakke and Van Pelt, 1970). The gradients were allowed to diffuse overnight at 4°C. A volume equal to that of the samples was removed from each gradient before loading the samples. The gradients were centrifuged at 185,000 *g* (39k rpm) for 5 hr at 15°C with a Beckman SW41 rotor. Gradient zones containing RNA were collected

using an ISCO UV fractionator (ISCO, Inc., Lincoln, NE). The RNA was precipitated overnight at -20°C by adding 1/20 volume of 3 M sodium acetate (pH 5.2), and 3 volumes of 100% cold ethanol. After centrifugation at 10,000 *g* for 10 min, the pellet was rinsed with 70% ethanol and vacuum-dried. The RNA was then resuspended in a small volume of sterile water and stored at -80°C.

The viral RNA was also extracted from incubated virus preparations by adding an equal volume of phenol/chloroform (1:1) to the mixture, inverting gently and centrifuging at 12,000 *g* in an Eppendorf microcentrifuge for 5 min. The phenol fraction was removed by adding an equal volume of chloroform, and centrifuging. The RNA was then precipitated with 3 volumes of 100% ethanol in the presence of 0.3 M sodium acetate (pH 5.2) at -20°C overnight. After centrifugation, the RNA was resuspended in sterile water and stored at -80°C.

Synthesis of cDNAs

Two types of cDNAs were synthesized, one with oligo$(dT)_{12\text{-}18}$ primers and the other with random hexamers. Freshly prepared viral RNA was used for cloning, with 5 µg RNA being used as templates for the first strand synthesis. The first and second strand cDNAs were synthesized using a TimeSaver™ cDNA synthesis Kit (Pharmacia Biotech., Inc., Piscataway, NJ) following the manufacturer's instructions. The first-stranded cDNA synthesis was labeled with 1 µl (10 µCi α-^{32}P-dCTP) (3000 Ci/mmol) (Du Pont NEN, Boston, MA) and used as a tracer. After the double-stranded cDNA synthesis, the sample was extracted with phenol-

chloroform and purified by passing through a Sepharose CL-4B column. The next step involved ligation of *Eco*RI/*Not*I linkers to cDNAs.

In vitro packaging of the λDNA was performed using the Packagene Lambda DNA Packaging System under the conditions recommended by the manufacturer (Promega Co., Madison, WI). The phage titer was determined by plating small aliquots of the packaging extract on XL1-Blue cells.

Size analysis of cDNA was performed on a 0.9% gel. The gel was exposed to X-ray film and compared to a 1-kb ladder molecular weight standard (Gibco BRL).

Immunoscreening of DsMV-Ch1 Phage Clones

Immunoscreening for clones expressing CP or CI genes of the DsMV genome was conducted essentially according to manufacturer's instructions as described in the *Pico*Blue™ Immunoscreening Kit and Predigested Lambda ZAP II/*Eco*RI/CIAP Cloning Kit (Stratagene, La Jolla, CA). The titered bacteriophage library (500 pfu/plate) was used to inoculate 200 µl of freshly prepared XL1-Blue of *E. coli* competent cells (OD_{600} = 0.5), and incubated at 37°C for 15 min. The mixture was added to 3 ml of NZY top agar (0.5% NaCl, 0.2% $MgSO_4 . 7H_2O$, 0.5% yeast extract, 1% casein hydrolysate, and 0.8% agar) containing 500 µg/ml X-galactoside and 10 mM isopropyl-ß-D-thiogalactopyranoside (IPTG), which was poured onto a NZY plate (80 mm x 80 mm) and incubated at 37°C for 5-6 hr to allow for formation of plaques (0.5 mm in diameter). The plates were chilled at 4°C for 2 hr in order to prevent the top agar from sticking to the nitrocellulose membranes. The plates were dried in a hood for 15

min, and sterile nitrocellulose membranes soaked in 10 mM IPTG were carefully layered upon them. The plates were incubated at 42°C for an additional 4-5 hr to allow expression of the cloned gene(s). IPTG is a gratuitous inducer used to induce the expression of the ß-galactosidase fusion protein, and λZAP II phage is a temperature-sensitive mutant (Pharmacia Biotech.). The membranes were lifted, rinsed three times for 10 min each in TBST solution, and processed as described in western blot analysis in chapter 4. DsMV-FL antiserum (1:1000) and DsMV-FL CI antiserum (1:1000) were employed as primary antibodies. Positive plaques were isolated with a sterile glass pipette and placed in 50 µl of SM buffer (100 mM NaCl, 50 mM Tris-HCl, pH 7.5, 10 mM $MgCl_2$) containing 3 drops of chloroform. The tubes were vortexed and incubated at room temperature for 2 hr to allow the phages to diffuse into the solution. Forty µl aliquots of the phage clones were added to 200 µl of the XL1-Blue cells (OD_{600} = 0.5), absorbed at 37°C for 15 min, and amplified at 37°C in a shaker overnight in 5 ml of 2X YT with 0.2% maltose and 10 mM $MgCl_2$. The cultures were centrifuged at 2,000 *g* for 10 min to remove the cell debris. Supernatants containing phages were stored at 4°C with addition of chloroform to a final concentration of 5%.

In Vivo Excision of DsMV-Ch1 Plasmids from The λZAP II Vector

The plasmid clones were excised from the λZAP II vector according to the manufacturer's instructions using a Predigested λZAP II/*Eco*RI/CIAP Cloning Kit (Stratagene) with the following modification except that amplified phage clones (200 µl) were used to start the excision. After excision, plasmids (pDCPn or pDCIn) were transformed into

SOLR of *E. coli* cells, and plated on LB/AMP plates (0.5% NaCl, 1.0% tryptone, 0.5% yeast extract, 1.5% agar, and 500 µg/ml). Single colonies were picked up, amplified and used for clone analysis.

Analysis of DsMV-Ch1 Clones:

Plasmids were purified according to a mini-prep procedure described by QIAGEN Inc. (Chatsworth, CA). Up to 1.5 ml of cell culture was collected by centrifugation in a microcentrifuge at 12,000 *g* for 1 min at 4°C. The supernatant was discarded, and the pellet was resuspended in 320 µl of P1 buffer (50 mM Tris-HCl, pH 8.0, 10 mM EDTA, RNase A 100µg/ml). After incubation at room temperature for 5 min, 320 µl of P2 (200 mM NaOH, 1% SDS) was added and the tube was mixed gently by inversion. After adding 320 µl of P3 buffer (3 M potassium acetate, pH 5.5), the tube was centrifuged at 12,000 *g* for 5 min. The aqueous phase was transferred to a fresh tube and 1 volume of 100% isopropanol was added. The mixture was then centrifuged at 12,000 *g* for 5 min. The pellet was resuspended in 25 µl of sterile water, and then screened by *Eco*RI (Promega) digestion for clone with a single insert to be used for sequence analysis.

DNA Sequencing of DsMV-Ch1 Clones

The plasmid preparations were sequenced by the dideoxy chain termination procedure using the Sequenase Version 2.0 DNA Sequencing Kit (United States Biochemical, Cleveland, OH) with α-^{35}S-dATP. T7 and T3 primers complementary to the pBlueScript vector and

synthesized internal primers were used to complete the sequence determination for either two clones or both strands of the same clone. Sequencing products were performed in a 6% (w/v) polyacrylamide gel containing 7 M urea. Autoradiographs of air-dried sequencing gels were made using XAR x-ray film (Eastman Kodak, Rochester, NY).

Pairwise Comparison and Phylogenetic Analysis

Sequence analysis and comparisons were made using the University of Wisconsin Genetics Computer Group (GCG) Sequence Software package version 7.0 (Devereux et al., 1984) available at the University of Florida ICBR Biological Computing Facility. The sequences of the 3' non-coding region, CP, and NIb proteins of DsMV isolates were compared to 14 other potyviruses. The sequences of 14 other potyviruses and DsMV-LA were obtained by Farfetch from GeneBank, and the sequences were aligned using the Pileup method of aligning multiple sequences in the GCG program.

Phylogenetic analyses were done by a cladistic parsimony method using the computer program PAUP version 3.1.1 developed by D. L. Swoford (distributed by the Illinois Natural History Survey, Champaign, Ill). Optimum trees were obtained with the heuristic method with the tree-bisection-reconnected branch-swapping option or exhaustive method. One hundred bootstrap replications were performed to establish confidence estimates on groups contained in the most parsimonious tree.

Aphid Transmission

Three caladium isolates (Ch1, Ch2 and Ch3) were tested for aphid transmission. The aphids (*Myzus persicae*), maintained on pepper (*Capsicum annuum*), were starved for two hr and then placed on infected leaves for 30-40 sec. The aphids were then moved to *P. selloum* seedlings used as test plants. Each trial consisted of 6 test plants and each test plant received 10 aphids. After 15 min the aphids were killed. The plants were maintained in a greenhouse, and two weeks later, were observed for symptom expression. Visual observations were confirmed by I-ELISA test using DsMV-FL antiserum.

Subcloning and Expression of the DsMV Coat Protein and NIb protein

Based on the CP nucleotide sequence of DsMV-Ch1, two primers, namely EH232 (5'-AAGCTTGCAGGCTGATGATACAG-3') corresponding to the 5'-end of the CP gene and EH234 (5'-GAATTCTTGAACACCGTGCAC-3') corresponding to the 3'-end of the non-coding region, were synthesized at the University of Florida DNA Synthesis Core. A *Hind*III or *Eco*RI restriction site was included at the 5'-end of each primer for directional cloning of the CP gene into an expression pETh-3 vector (McCarty et al., 1991) at *Hind*III and *Eco*RI on the polylinker.

The intact CP gene (942 nt) was amplified by PCR as described in chapter 4. The DNA fragment was purified from a 0.9% agarose gel by using Prep-A-Gene Master Kit (Bio-Rad) according to manufacturer's instructions. The purified DNA was then cloned into a PCR vector pGEM T vector (Promega) to generate pGEM-T-CP according to the manufacturer's

instructions. The plasmid pGEM-T-CP was digested by *Hind*III and *Eco*RI (Promega) and subcloned into *Hind*III/*Eco*RI double-digested pETh-3 to generate pETh-3-CP. The nucleotide sequence of the vector/insert junction was confirmed by DNA sequencing using the Sequenase Version 2.0 DNA Sequencing Kit (US Biochemical).

A single colony culture (5 ml) of *E. coli* BL21DE3pLysS, transformed with pETh-3-CP, was grown overnight at 37°C in LB containing 50 µg/ml ampicillin and 25 µg/ml chloramphenicol. The overnight culture was diluted 1:100 into 5 ml or 250 ml (large scale) of M9 medium (Sambrook et al., 1989) containing 50 µg/ml ampicillin, 25 µg/ml chloramphenicol, 0.4% glucose and 0.5% tryptone, and the culture was shaken at 37°C until early log phase (OD_{600} = 0.6). Then IPTG was added to a final concentration of 1 mM, and growth was continued for an additional 4 hr at 37°C. *E. coli* BL21DE3pLysS cells were harvested by centrifugation at 5,000 *g*, the culture broth was discarded, and the cell pellet was resuspended in one tenth of the original volume of TE buffer (10 mM Tris-HCl, pH 8.0, 1 mM EDTA) and frozen overnight at -20°C. The viscous cell suspension was thawed, sonicated for 30 sec and the lysate was then centrifuged at 10,000 *g* for 10 min. The pellet was resuspended in a small volume of TE buffer. The preparations were then analyzed by SDS-PAGE. Both noninduced pETh-3-CP and pETh-3 cultures were tested as controls.

The same approach used for the DsMV CP expression was applied to express the NIb polymerase in *E. coli*. Three pairs of synthesized primers were used for PCR amplification of either the intact or truncated NIb gene or a fragment containing the C-terminal region of the NIa and the NIb gene: EH239 (5'-GAATTCATGCAAAGTGGGTGGGTGA-3')

corresponding to the 5'-end of the NIb gene and EH238 (5'-AGATCTCTACTGCAACACAACCTC-3') corresponding to the 3'-end of the NIb gene, primers EH256 (5'-AAGCTTGCAGCGAGATGATGA-3') corresponding to a sequence in the NIb gene and EH238, primers EH267 (5'-GGGATTGGAATAGGCT-3') corresponding to a sequence in the 5'-terminal region of the NIa gene and EH238.

Antigen Preparation and Antibody Production

After sonication, the fusion protein expressed by *E. coli* was partially purified by three cycles of centrifugation at 10,000 *g*, washing each pellet with TE buffer, and separating the proteins by preparative SDS-PAGE. Protein bands were visualized by incubating gels in 0.2 M KCl for 10 min at 4°C. The targeted protein band was excised, washed three times in cold deionized water, and frozen at -20°C. The cut pieces of the gel were then eluted using a Bio-Rad Electoelutor at 10 mAmp/tube with constant current for 5 hr. The extracts containing the protein were collected and dialyzed overnight against distilled water at room temperature. Purity of the eluted protein was checked by analytical SDS-PAGE, after which the protein was lyophilized.

Immunization was conducted as described by Purcifull and Batchelor (1977). A quarter ml of purified protein (1 mg) at 4 mg/ml was emulsified with an equal volume of complete Freund's adjuvant and injected into the thigh muscles of a New Zealand white rabbit (No. 1210). This was followed by two 1.0 mg injections of the CP protein emulsified with incomplete Freund's adjuvant two or three weeks later. Blood was collected weekly for two

months, starting two weeks after the third injection. After a four week interval, a booster injection was given, followed by subsequent bleeding.

Serological Evaluation of Antiserum

The antiserum obtained was tested by SDS-immunodiffusion, I-ELISA and Western blotting analysis. The SDS-immunodiffusion tests were conducted using crude extracts as described by Purcifull and Batchelor (1977). The immunodiffusion medium consisted of 0.5% Noble agar, 1% sodium azide and 0.5% SDS. About 12 ml of the thoroughly mixed agar suspension was poured into a disposable plastic petri dish (90 mm x 15 mm). A set of wells consisting of six peripheral antigen wells surrounding a central antiserum well with an interval of 5 mm from the edges of one another were made with a gel cutter. Samples were prepared by grinding plant tissues in 1% SDS solution (final concentration) with a mortar and pestle. After addition of the antigens and antisera, the double radial diffusion plates were incubated at 25°C. Results were recorded after 24 hr and 48 hr. The DsMV-FL antiserum was used as a control. Both I-ELISA and Western blotting using the DsMV-FL antiserum and the expressed DsMV CP antiserum were conducted using procedures described in Chapter 4.

Results

Purification of DsMV-Ch and Isolation of Viral RNA

The DsMV virions were only purified from the first two newly formed leaves after inoculation of *P. selloum* seedlings grown in relatively cool weather conditions during the

spring. The A_{260}/A_{280} ratio of the final purified virus preparations obtained by 2 mM HEPES buffer in 2 trials ranged from 1.11 to 1.20, with an average of 1.16. The estimated virus yields were 4-8 mg/100g leaf tissue. SDS-PAGE analysis showed that the amount of virus obtained by ultracentrifugation was slightly higher than that obtained by PEG precipitation (Fig. 2-1A). The leaf tissues had higher concentrations of the virus than the root tissue (Fig. 2-1B). Although many DsMV particles were seen in the leaf dip preparations, the virus could not be purified from infected leaves of caladium, calla lily, cocoyam, and taro, presumably due to the presence of viscous host components, probably polysaccharides.

Although cesium chloride was used in the purification of DsMV in previous research (Abo El-Nil et al., 1977; Kositratana, 1985; Shimoyama et al., 1992), DsMV was degraded in cesium chloride gradients made in 20 mM HEPES, pH 8.0, containing 10 mM EDTA and 0.1% β-ME. Electron microscopic examination of the virus preparations revealed numerous fragments of virus particles. It was presumed, therefore, that degradation of virus particles resulted from cesium chloride. In cesium sulfate gradients, however, two opalescent virus bands were formed, and the bottom band contained more virions than the top band based on the SDS-PAGE result (Fig. 2-1B). Furthermore, the virions from the bottom band collected from the cesium sulfate gradients were less degraded than those noted in cesium chloride gradients based on electron microscopy (Fig. 2-2).

Viral RNA was readily extracted in phenol/chloroform, and the procedure required a minimum of time to process, thereby reducing the chance of degradation. The yield of viral RNA extracted by phenol/chloroform was 14.5 μg, which was much higher than that by

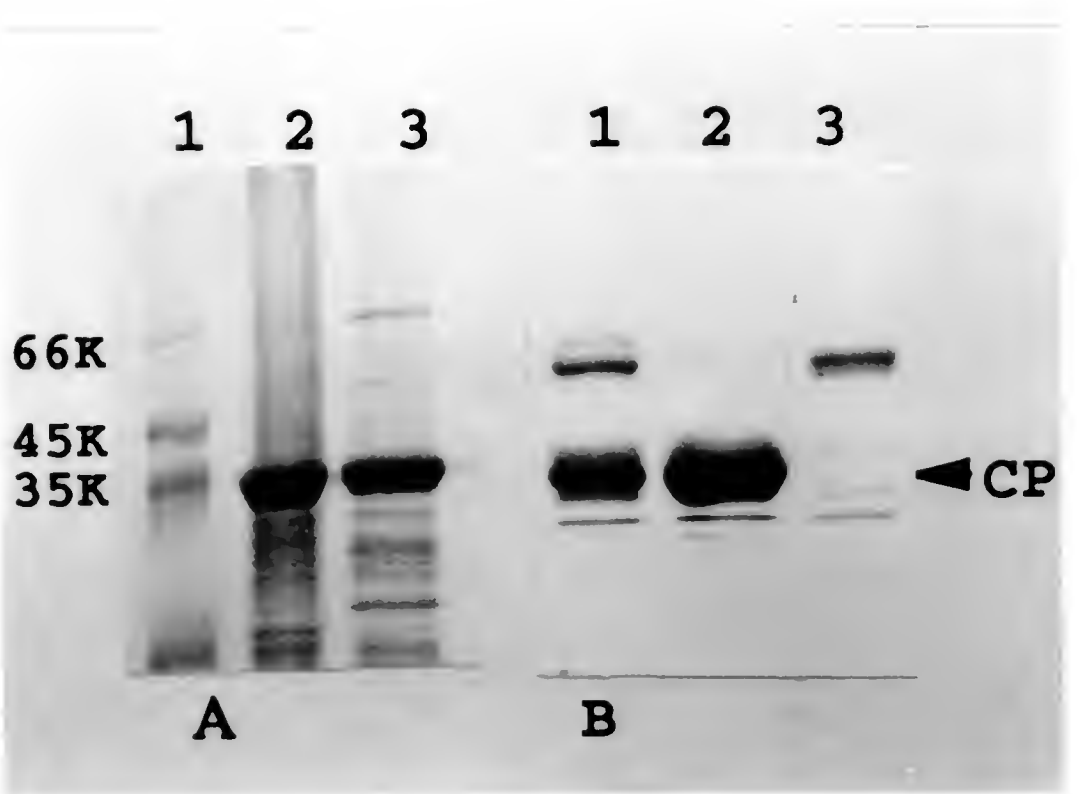

Fig. 2-1. Analysis of partially purified and purified DsMV-Ch1 preparations. A. The partially purified preparations. Lane 1, protein standards: bovine serum albumin (66 kDa), egg albumin (45 kDa), pepsin (35 kDa); lane 2, the purified preparation using the ultracentrifugation method; lane 3, the purified preparation using PEG precipitation. B. The purified preparations. Lane 1, the viral preparation collected from the top band of the Cs_2SO_4 gradient; lane 2, the viral preparation collected from bottom band of the Cs_2SO_4 gradient; lane 3, the preparation from root tissues of *P. selloum* seedlings. The virus was purified from inoculated *P. selloum* seedlings as procedures described in the text. Protein samples were analyzed by 10% sodium dodecyl sulfate polyacrylamide gel electrophoresis followed by Coomassie staining. The coat protein of the purified DsMV-Ch1 is indicated by the arrow.

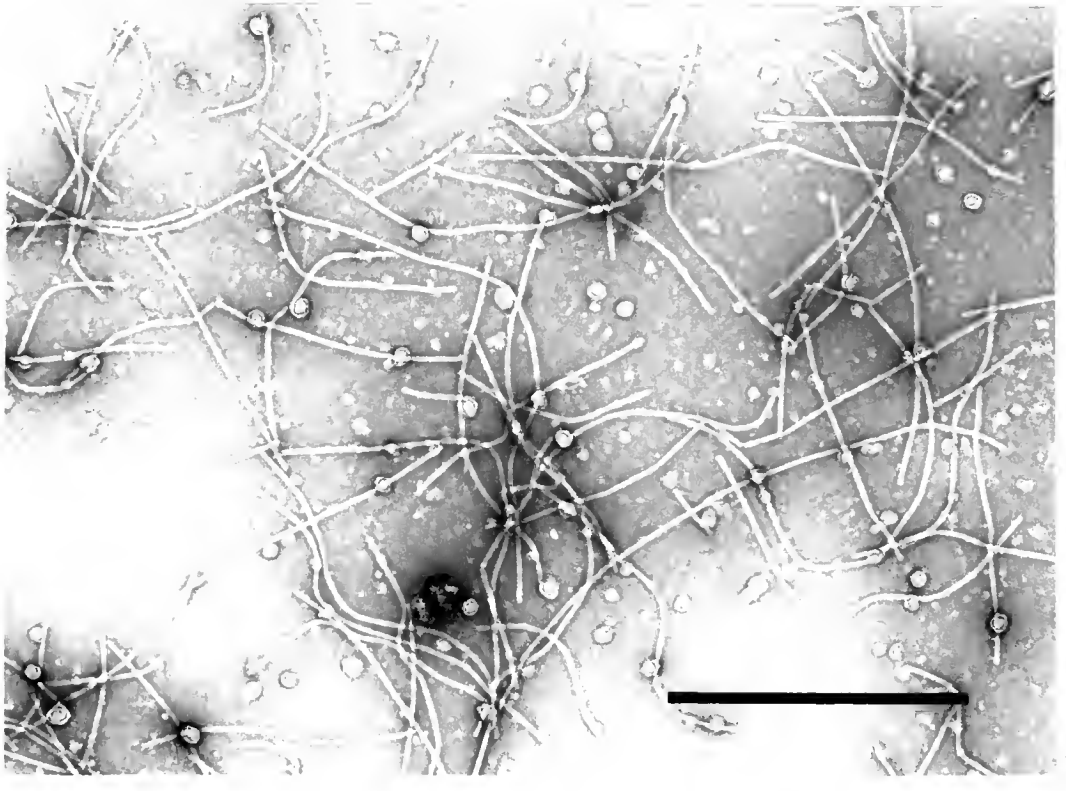

Fig. 2-2. Electron micrograph of a purified DsMV-Ch1 preparation negatively stained with 2% uranyl acetate. The virus was purified from inoculated *P. selloum* seedlings by ultracentafugation purification method described in text. Bar = 500 nm.

sucrose gradient (5.28 µg), and the resulting RNA was much more intact in agarose gel (Fig. 2-3).

Molecular Analysis of cDNA Clones

After serological screening with DsMV-FL virion antiserum, 16 clones expressing the CP (pDCP) were selected and screened by *Eco*RI and *Not*I digestions. Twelve pDCP clones with a single insert were selected for direct sequence mapping (Table 2-1). The sizes of the inserts in these clones ranged from 1.1-5.2 kb. The reaction of DsMV-FL CI antiserum was nonspecific, and 22 white clones (pDCI) were randomly selected for *Eco*RI and *Not*I digestion screening and sequence mapping. Among these clones, eight had correct inserts, which ranged from 0.9 to 2.7 kb (Table 2-2). These cDNA clones with overlapping inserts covered all but the 1.2 kb central region and the upstream 5'-terminal region of the DsMV-Ch1 genome, based on comparisons with the published sequence of SbMV (Jayaram et al., 1992) (Fig. 2-4). Twelve clones with insert sizes ranging from 1.1 to 3.4 kb were selected for sequence analysis (Fig. 2-5).

Sequences Representing the 3'-terminal region of DsMV-Ch1

The nucleotide sequence of 3158 bases (excluding the poly(A) tail) corresponding to the 3'-terminal region of DsMV RNA was determined (Fig. 2-6). The nucleotide positions were confirmed by analyzing either both strands of the same clones or individual strands of different clones. Stretches of 20-40 adenosine residues were found at the ends of two clones

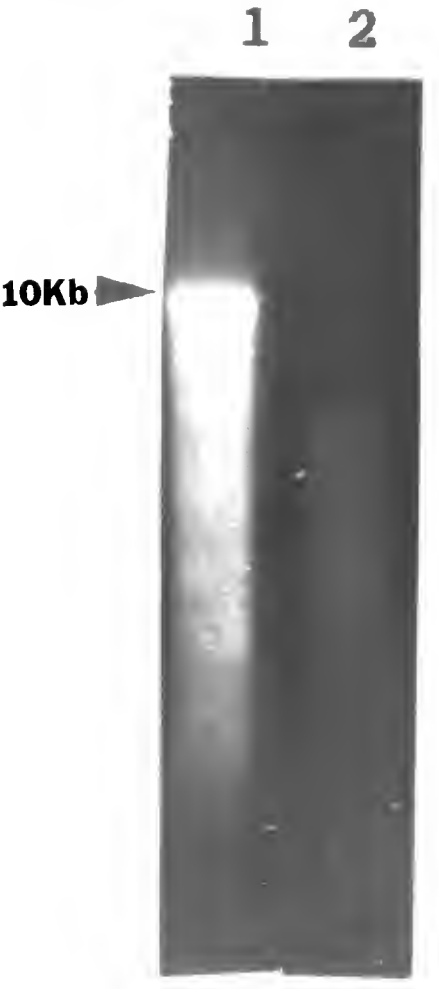

Fig. 2-3. Agarose gel electrophoresis of DsMV-Ch1 RNA isolated from purified virions: Lane 1, viral RNA isolated by phenol/chloroform extraction; lane 2, viral RNA isolated by sucrose gradient. The viral RNA was loaded onto a 0.9% agarose gel. The separated RNA was stained by ethidium bromide and visualized under UV light.

Table 2-1. DsMV-Ch1 cDNA clones identified by immunoscreening[1] and preliminary sequencing[2]

Clone designation	Approximate size (kbp)	Serological reactivity[3]
pDCP1	2.0	CP
pDCP2	5.2	CP
pDCP3	4.2	CP
pDCP5	1.1	CP
pDCP6	2.8	CP
pDCP7	1.4	CP
pDCP8	2.5	CP
pDCP10	2.1	CP
pDCP11	2.5	CP
pDCP12	2.9	CP
pDCP14	1.9	CP
pDCP15	2.2	CP
pDCP16	1.2	CP

[1] cDNA library in λZAP II vectors was plaque screened by DsMV- FL antiserum. White plaques expressing the CP were selected.

[2] The selected clones were confirmed by *Eco*RI digestion, and the clones with a single insert were selected. The positive clones were sequenced by vector primers, T3 and T7, and results were mapped to relative positions in the RNA genome compared to a published nucleotide sequence of SbMV by a Gap program of the GCG program package.

[3] Reactive with DsMV-FL antiserum.

Table 2-2. DsMV-Ch1 cDNA clones identified by preliminary sequencing[1]

Clone designation	Approximate size (kbp)	Relative position[2]
pDCI2	1.8	NIb, CP
pDCI4	1.3	HC-Pro, CI
pDCI7	0.9	HC-Pro
pDCI18	1.5	NIb
pDCI19	2.7	HC-Pro, CI
pDCI21	1.8	P1, P2
pDCI22	1.6	P1, P2

[1] cDNA library in λZAP II vectors was plaque screened by DsMV-FL CI antiserum. White clones were selected. The selected clones were confirmed by *Eco*RI digestion, and the clones with a single insert were selected.

[2] The positive clones were sequenced by vector primers, T3 and T7, and the results were mapped to relative positions in the RNA genome compared to a published SbMV nucleotide sequence by a Gap program of the GCG program package.

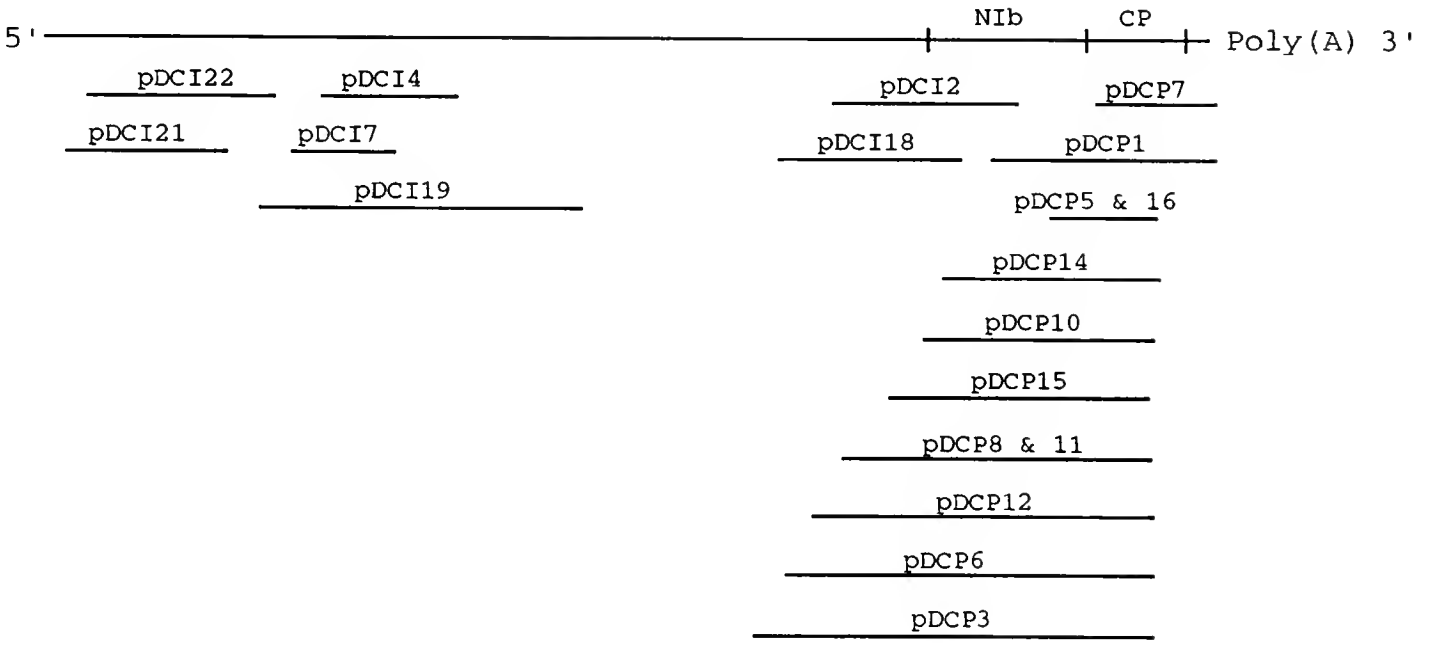

Fig. 2-4. Map of the cDNA clones representing the DsMV-Ch1 genome. The position of each clone was mapped by preliminary sequencing and comparing them with the published nucleotide sequence of SbMV (Jayarama et al.,1992)

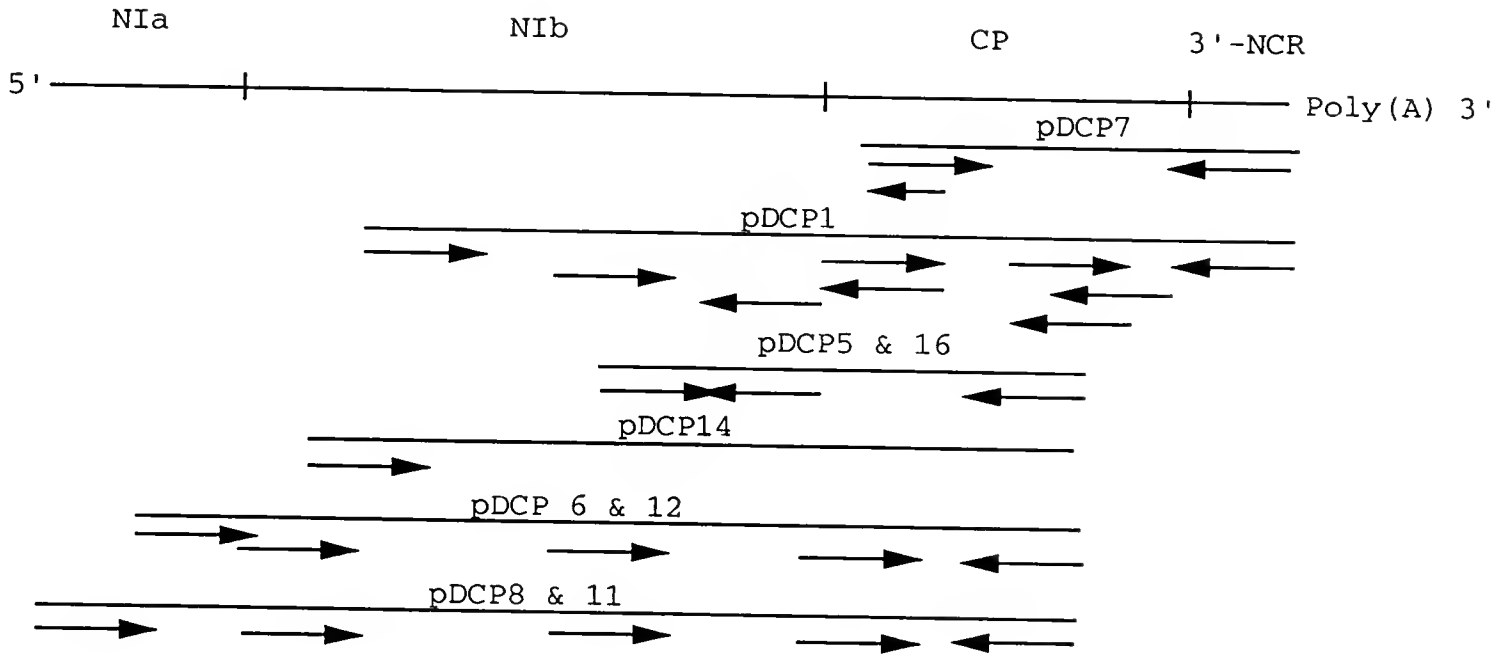

Fig. 2-5. Sequencing strategy used for the cDNA clones representing the 3'-terminal region of DsMV-Ch1. Arrows indicate the direction of sequencing and distance read from the beginning of the clone from the location of a primer. NIa = NIa protease gene; NIb = polymerase gene; CP = coat protein gene; 3'-NCR = 3' non-coding region.

Fig. 2-6. Nucleotide sequence of the 3'-terminal region of DsMV-Ch1. The predicted amino acid sequence of the open reading frame coding for the putative polyprotein is shown. Underlined amino acids correspond to the NIa protease activity site (between residues 31 and 34), to the polymerase active motif (between 451 and 495), and aphid transmission motif (amino acids 662-664). Amino acids underlined with dotted lines and slashes (/) correspond to the putative polyprotein cleavage sites.

```
   1 CAAAGAGAGAGGAGCGCGTATGCATGGTTGGCACGAATTTCCAAGATAAAAGCATGCGCG  60
       K  R  E  E  R  V  C  M  V  G  T  N  F  Q  D  K  S  M  R  A

  61 CTACGATCTCGGAAGCATCTCTCATTCTACCAGAAGGGCAGGGAACATTCTGGAAACATT 120
       T  I  S  E  A  S  L  I  L  P  E  G  Q  G  T  F  W  K  H  W

 121 GGATTTCAACTAAGGATGGGGAGTGTGGGATCCCCATGGTGGCTGTGAGTGATGGATATA 180
       I  S  T  K  D  G  E  C  G  I  P  M  V  A  V  S  D  G  Y  I

 181 TAGTTGGTTTCCATGGCCTTGGCTCAAACATATCTGAGAGGAACTATTTTGTCCCTTTCA 240
       V  G  F  H  G  L  G  S  N  I  S  E  R  N  Y  F  V  P  F  T

 241 CTGACGACTTCGAGCAAACACATCTTAAAAGGCTCGATAGTCTTGAATGGACCCAGCATT 300
       D  D  F  E  Q  T  H  L  K  R  L  D  S  L  E  W  T  Q  H  W

 301 GGCACTTTCAGCCTGACAAGATAGCTTGGGGTTCACTCAGACTAGTTAATGACCAACCTA 360
       H  F  Q  P  D  K  I  A  W  G  S  L  R  L  V  N  D  Q  P  T

 361 CTGAAGATTTTAAGATTTCAAAGTTAATTTCAGACCTTTTCGAAAATCCTGTACAATTAC 420
       E  D  F  K  I  S  K  L  I  S  D  L  F  E  N  P  V  Q  L  Q/

 421 AAGGGTCTCAAAGTGGGTGGGTGATTAATACTGCCGAAGGGAATCTAAAAGCTGTTGCCC 480
       G  S  Q  S  G  W  V  I  N  T  A  E  G  N  L  K  A  V  A  R

 481 GGTGTGAAAGTGCACTCGTGACAAAACATACAGTGAAGGGACCGTGCAGATACTTCTCAG 540
       C  E  S  A  L  V  T  K  H  T  V  K  G  P  C  R  Y  F  S  E

 541 AGTACTTGAGTTCAAACCAGGAAGCTGAAAAGTTTTTCAGACCATTCATGGGAGCTTATC 600
       Y  L  S  S  N  Q  E  A  E  K  F  F  R  P  F  M  G  A  Y  R

 601 GCTCAAGTAGACTTAACAGGGAAGCTTTCAAGAAAGACTTCTTTAAGTATGCAAAGCCTG 660
       S  S  R  L  N  R  E  A  F  K  K  D  F  F  K  Y  A  K  P  V

 661 TTGAGTTGAATAAAGTTGATTTCAATGCCTTCCAGATTGCAGTGGCAAGTGTGGAGACAA 720
       E  L  N  K  V  D  F  N  A  F  Q  I  A  V  A  S  V  E  T  M

 721 TGATGATGGAAACAGGATTTAGCGAATGTGAGTATATCACAGACGCTCAAACAATCATTG 780
       M  M  E  T  G  F  S  E  C  E  Y  I  T  D  A  Q  T  I  I  D

 781 ACTCCCTCAATATGAAAGCAGCTGTGGGGGCTCAATATCGTGGGAAGAAGTCTGAGTACT 840
       S  L  N  M  K  A  A  V  G  A  Q  Y  R  G  K  K  S  E  Y  F

 841 TCCACGATATGGAGGTCTACGATATGGAACGACTCCTCTTCCAAAGTTGTGAAAGACTAT 900
       H  D  M  E  V  Y  D  M  E  R  L  L  F  Q  S  C  E  R  L  F

 901 TCTATGGGAAGAAGGGAGTCTGGAATGGCTCATTAAAGGCAGAGTTGCGTCCAATTGAGA 960
       Y  G  K  K  G  V  W  N  G  S  L  K  A  E  L  R  P  I  E  K

 961 AAACGCAACTCAATAAAACAAGGACATTTACTGCCGCTCCTCTTGACACATTGTTAGGAG 1020
       T  Q  L  N  K  T  R  T  F  T  A  A  P  L  D  T  L  L  G  A

1021 CGAAGGCTTGTGTGGATGACTTTAACAACCAATTCTATAGTCTCAACCTAAAGTGTCCAT 1080
       K  A  C  V  D  D  F  N  N  Q  F  Y  S  L  N  L  K  C  P  W
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Fig. 2-6--continued

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1081 GGACAGTTGGTATGACCAAATTTTATAAAGGTTGGGATTCATTGATGAGAAAGCTTCCAG 1140
       T  V  G  M  T  K  F  Y  K  G  W  D  S  L  M  R  K  L  P  E

1141 AAGGATGGGTCTACTGCCACGCTGATGGGTCTCAGTTTGACTCATCATTAACACCACTCC 1200
       G  W  V  Y  C  H  A  D  G  S  Q  F  D  S  S  L  T  P  L  L

1201 TCATAAACGCGGTCGTGGACATCAGGAAGTTTTTCATGGAGGAGTGGTGGGTTGGTGAAG 1260
       I  N  A  V  V  D  I  R  K  F  F  M  E  E  W  W  V  G  E  E

1261 AAATGCTTGACAACTTGTATGCTGAAATTGTCTACACACCTATATTGACCCCAGATGGAA 1320
       M  L  D  N  L  Y  A  E  I  V  Y  T  P  I  L  T  P  D  G  T

1321 CAATTTTTAAGAAATTTAGGGGCAATAATAGTGGACAACCATCGACAGTCGTGGATAATA 1380
       I  F  K  K  F  R  G  N  N  S  G  Q  P  S  T  V  V  D  N  T

1381 CATTGATGGTTGTCATTTCAGTTTACTACGCATGTATCAAGCAAGGTTGGACGGATTATG 1440
       L  M  V  V  I  S  V  Y  Y  A  C  I  K  Q  G  W  T  D  Y  D

1441 ATGTTAGTCAAAGAATAGTCTTCTTTGCAAATGGTGATGACATCATATTGGCTGTGCAGC 1500
       V  S  Q  R  I  V  F  F  A  N  G  D  D  I  I  L  A  V  Q  R

1501 GAGATGATGAACCCATCCTTAATACCTTTCAGGATTCTTTTCACGAATTGGGGCTCAACT 1560
       D  D  E  P  I  L  N  T  F  Q  D  S  F  H  E  L  G  L  N  Y

1561 ATGATTTCTCTGAGCGCACGATGAAGAGAGAGGAACTTTGGTTCATGTCCCATCAAGCTA 1620
       D  F  S  E  R  T  M  K  R  E  E  L  W  F  M  S  H  Q  A  M

1621 TGAAAGTAGGGGATGTTTATATCCCTAAACTAGAGCGAGAGAGAATTGTATCAATTTTAG 1680
       K  V  G  D  V  Y  I  P  K  L  E  R  E  R  I  V  S  I  L  E

1681 AATGGGATAGAAGCAAAGAAATCATGCACAGAACAGAGGCAATTTGTGCAGCTATGATAG 1740
       W  D  R  S  K  E  I  M  H  R  T  E  A  I  C  A  A  M  I  E

1741 AAGCATGGGGTTACACTGACCTCTTGCAAGAAATAAGGAAATTCTATCTATGGCTGCTTG 1800
       A  W  G  Y  T  D  L  L  Q  E  I  R  K  F  Y  L  W  L  L  E

1801 AAAAAGATGAATTTAAGACACTAGCCTCTGAAGGGCGGGCACCATATATTGCTGAAACAG 1860
       K  D  E  F  K  T  L  A  S  E  G  R  A  P  Y  I  A  E  T  A

1861 CACTCAAGAAGCTATACACAGATGAAAACATAAAGGAGTGCGAGCTTCAGCGTTATCTGG 1920
       L  K  K  L  Y  T  D  E  N  I  K  E  C  E  L  Q  R  Y  L  D

1921 ATGCTTTCAATTTTGAAATGTTCTGCGAACATGATGAGGTTGTGTTGCAGGCTGATGATA 1980
       A  F  N  F  E  M  F  C  E  H  D  E  V  V  L  Q/ A  D  D  T

1981 CAGTTGATGCAAGGAAAAACAACAATACTACAAAAACAACTGAAACAAAAACACCTGCAA 2040
       V  D  A  R  K  N  N  N  T  T  K  T  T  E  T  K  T  P  A  T

2041 CGGGTGGTGGGAACAACACAAACAACAACACGCCACCTGTAGATAACACAACCAACAATA 2100
       G  G  G  N  N  T  N  N  N  T  P  P  V  D  N  T  T  N  N  N

2101 ATCCTCCACCGCCACCACCGGCGGTTACAAAGGTAACAGAGGTACCCGCCAATAAGCAAG 2160
       P  P  P  P  P  P  A  V  T  K  V  T  E  V  P  A  N  K  Q  V
```

Fig. 2-6--continued

```
2161 TGGTCCCAGCAGCAAGTGAGAAAGGTAAGGAAGTTGTGAAAGATGTTAACGCTGGCACTA 2220
       V  P  A  A  S  E  K  G  K  E  V  V  K  D  V  N  A  G  T  S

2221 GTGGCACATACTCCGTACCTCGGTTGAATAGAATCACAAACAAAATGAATTTACCTTTAG 2280
       G  T  Y  S  V  P  R  L  N  R  I  T  N  K  M  N  L  P  L  V

2281 TTAAAGGTAAATGCATTTTAAATTTGAATCATTTAATCGAGTACAAGCCAGAACAGCGTG 2340
       K  G  K  C  I  L  N  L  N  H  L  I  E  Y  K  P  E  Q  R  D

2341 ACATATTCAATACCAGAGCCACCCACACTCAATTTGAAGTCTGGTACAATGCTGTCAAGA 2400
       I  F  N  T  R  A  T  H  T  Q  F  E  V  W  Y  N  A  V  K  R

2401 GAGAATACGAGCTTGAGGATGAGCAGATGCACATAGTTATGAATGGTTTTATGGTTTGGT 2460
       E  Y  E  L  E  D  E  Q  M  H  I  V  M  N  G  F  M  V  W  C

2461 GCATCGATAATGGAACATCACCTGATATCAACGGGGCTTGGGTGATGATGGACGGAAACG 2520
       I  D  N  G  T  S  P  D  I  N  G  A  W  V  M  M  D  G  N  D

2521 ATCAAATTGAATACCCGTTGAAGCCAATTGTTGAAAATGCAAAACCAACCTTGCGTCAGA 2580
       Q  I  E  Y  P  L  K  P  I  V  E  N  A  K  P  T  L  R  Q  I

2581 TAATGCATCACTTTTCTGACGCAGCAGAGGCATACATTGAACTGAGAAACGCAGAGAAAC 2640
       M  H  H  F  S  D  A  A  E  A  Y  I  E  L  R  N  A  E  K  P

2641 CGTATATGCCTAGATACGGTCTTATTCGCAATTTACGTGATGCAAGTCTCGCCCGGTATG 2700
       Y  M  P  R  Y  G  L  I  R  N  L  R  D  A  S  L  A  R  Y  A

2701 CTTTTGACTTTTATGAGGTCAATTCTAAAACACCGGTGCGAGCAAGAGAAGCAGTTGCGC 2760
       F  D  F  Y  E  V  N  S  K  T  P  V  R  A  R  E  A  V  A  Q

2761 AAATGAAGGCGGCTGCACTCTCTAACGTTACCACTAGGTTGTTTGGTTTGGATGGTAACG 2820
       M  K  A  A  A  L  S  N  V  T  T  R  L  F  G  L  D  G  N  V

2821 TTTCAACTTCAAGCGAGAACACTGAAAGGCACACTGCAAAAGACGTCACACCAAACATGC 2880
       S  T  S  S  E  N  T  E  R  H  T  A  K  D  V  T  P  N  M  H

2881 ACACTTTACTTGGTGTTTCGTCTCCGCAGTAAAGGTCTGGTAAACAGGGCCGACAGTTAT 2940
       T  L  L  G  V  S  S  P  Q  *

2941 TGGCTCGCTGTTTGTAGTTTTATTTATATAAAGTATTGTTTGTATTCAAGTAGTGCTATT 3000

3001 TGGTTATAAACTACAGCGTGGTTTTCCACCGATGTGGAGTTGGCTTTGCACCCTATTATC 3060

3061 TACGTCCTTTATGTATTTGAAAACTACTGAACTACTGCACCTACGTCAGACCGCAAGGCG 3120

3121 ATGGGCGCGGTAGGCGAGACGCTTCGTGCACGGTGTTCA(n) 3159
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Fig. 2-6--continued

(pDCP1 and pDCP7), suggesting this end represented the 3' end of the DsMV RNA. Computer analysis of the sequence revealed a unique large open reading frame (ORF) in the positive strand (virion sense). No other ORF of significant size was observed in either the plus or minus strands. The ATG start codon of the single ORF was not identified. However, by analogy with other potyviruses, it is presumably located near the 5'-terminus of the genome. The ORF terminated at the first TAA stop codon, which is located at 246 residues upstream of the 3' end. Nucleotide sequence heterogeneity among different cDNA clones was found to be about 98-99%.

The putative amino acid sequence includes 140 C-terminal amino acids of the NIa protease (about 40% of the protein), the NIb protein consisting of 516 amino acids, and the coat protein consisting of 313 amino acids (Fig. 2-6). The cleavage sites between these three proteins were identified by comparing them with those of other sequenced potyviruses (Dougherty et al.; 1988; Shukla et al., 1994). The cleavage site between the NIa and the NIb proteins was at the amino acid 140-141 position (Q/G), whereas the site between the NIb and the CP proteins was at the amino acid 656-657 position (Q/A). The conserved cleavage sequence VXXQ/A(G,S,E) has been found in both sites, which were VQLQ/G between the NIa and the NIb proteins and VVLQ/A between the NIb and the CP proteins.

Analysis of the NIb Protein

The NIb protein of DsMV contained the consensus sequence motif, SGXXXTXXXNT-(30aa)-GDD. This polymerase motif was found in the DsMV NIb protein

beginning at amino acid residue 451 of the sequence. A second consensus motif, YCHADGS, was present in the NIb protein at amino acid positions 384 to 390. The similarity of the DsMV NIb protein to those of other potyviruses ranged from 58% to 68% at the nucleotide level (Table 2-3), and from 72% to 85% at the amino acid level (Table 2-4), respectively. A phylogenetic tree was obtained from the alignment of putative NIb proteins of DsMV-Ch1 and 14 other potyviruses (Fig. 2-7) on the premise that this protein is the most conserved one among all the potyviruses (Shukla at al., 1994). Though DsMV was distinct from other potyviruses, it was closely related to ZYMV in the BCMV subgroup. The potyviruses, SbMV and WMV-2, were clustered together, confirming their close relationship (Shukla et al., 1994). It is interesting to note that all potyviruses except those in the BCMV subgroup were clustered together.

The putative amino acid sequence of the NIb protein of DsMV-Ch1 was used to prepare a hydrophobicity plot according to the method of Kyte & Doolittle (1982) (Fig.2-8). The N-terminal and C-terminal regions of this protein are hydrophilic, while in the central region there is an even distribution of hydrophilic and hydrophobic regions. Each of these regions consists of about 20 amino acids, suggesting the transmembrane property of the NIb protein.

Analysis of the Coat Protein

The coat protein of DsMV-Ch1 also showed a relatively high degree of similarity with those of other potyviruses (Table 2-5). The similarity ranged from 55% to 68% at the

Table 2-3. Percent nucleotide identity of NIb genes of DsMV-Ch1 and 14 other potyviruses

Virus	Percent similarity[2]													
	2	3	4	5	6	7	8	9	10	11	12	13	14	15
1. DsMV-Ch1	68	67	67	67	66	58	59	59	58	59	58	59	59	58
2. BCMV		60	72	73	70	60	59	58	58	59	59	59	59	61
3. SbMV			70	80	69	58	59	56	61	58	58	59	60	61
4. PStV				71	68	58	59	56	56	60	59	57	59	60
5. WMV-2					70	58	60	59	57	60	58	59	60	61
6. ZYMV						59	57	59	56	59	58	59	60	60
7. PVY-N							59	59	59	60	62	61	58	60
8. TEV								60	58	63	61	58	59	61
9. PSbMV									61	61	60	64	59	61
10. PRSV-P										59	60	60	59	62
11. TuMV											60	61	61	65
12. TVMV												60	60	62
13. SCMV													55	61
14. BYMV														61
15. PPV														

[1] Name and abbreviations of the viruses used in table: BCMV, bean common mosaic virus; BYMV, bean yellow mosaic virus; DsMV-Ch1, dasheen mosaic virus Ch1 isolate; PPV, plum pox virus; PSbMV, pea seed-borne mosaic virus; PRSV-P, papaya ringspot virus type P; PSbMV, pea seed-borne mosaic virus; PStV, peanut stripe virus; PVY-N, the N strain of potato virus Y; SbMV, soybean mosaic virus; SCMV, sugarcane mosaic virus; TEV, tobacco etch virus; TuMV, turnip mosaic virus; TVMV, tobacco vein mottling virus; WMV-2, watermelon mosaic virus 2; ZYMV, zucchini yellow mosaic virus.

[2] Percent similarity of NIb genes among the DsMV-Ch1 and other potyviruses was obtained by Pileup in the GCG program package.

Table 2-4. Percent similarity of the NIb protein, the coat protein and the 3' non-coding region (3'-NCR) of DsMV-Ch1 and 14 other potyviruses

Virus[1]	Percent similarity[2]		
	NIb	CP	3'-NCR
DsMV-Ch1	-	92	79
BCMV	85	82	36
SbMV	84	82	39
PStV	83	77	38
WMV-2	84	80	35
ZYMV	83	80	34
PVY-N	73	79	35
TEV	75	74	39
PSbMV	74	72	34
PRSV-P	73	72	34
TuMV	75	73	36
TVMV	72	69	33
SCMV	74	68	35
BYMV	76	70	36
PPV	74	66	34

[1] Name and abbreviations of the viruses used in table: BCMV, bean common mosaic virus; BYMV, bean yellow mosaic virus; DsMV-Ch1, dasheen mosaic virus Ch1 isolate; PPV, plum pox virus; PSbMV, pea seed-borne mosaic virus; PRSV-P, papaya ringspot virus type P; PSbMV, pea seed-borne mosaic virus; PStV, peanut stripe virus; PVY-N, N strain of potato virus Y; SbMV, soybean mosaic virus; SCMV, sugarcane mosaic virus; TEV, tobacco etch virus; TuMV, turnip mosaic virus; TVMV, tobacco vein mottling virus; WMV-2, watermelon mosaic virus 2; ZYMV, zucchini yellow mosaic virus.

[2] Percent similarity of the NIb, the CP and the 3'-NCR between the DsMV-Ch1 and other potyviruses was obtained by Gap in the GCG program package.

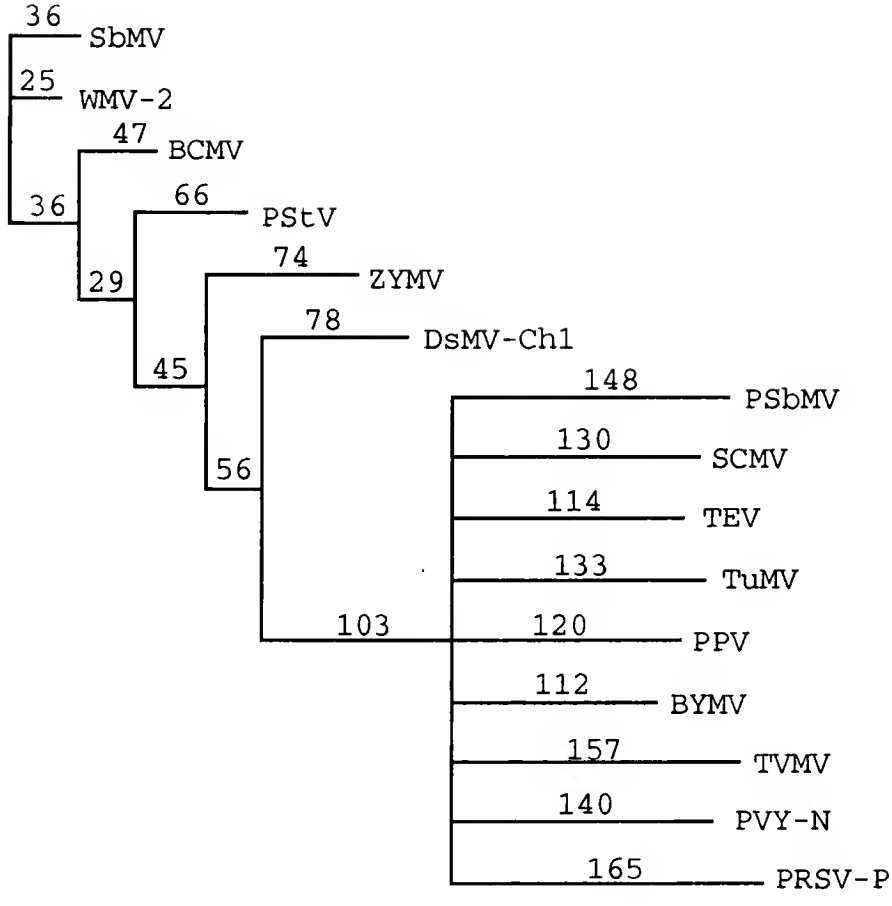

Fig. 2-7. Phylogenetic tree obtained from the alignment of putative polymerases between DsMV-Ch1 and 14 other potyviruses using the PAUP program. The tree is the bootstrap 50% majority-rule consensus tree. The number above a given branch refers to branch length. Vertical distances are arbitrary, and horizontal distances reflect number of amino acid differences between branch nodes.

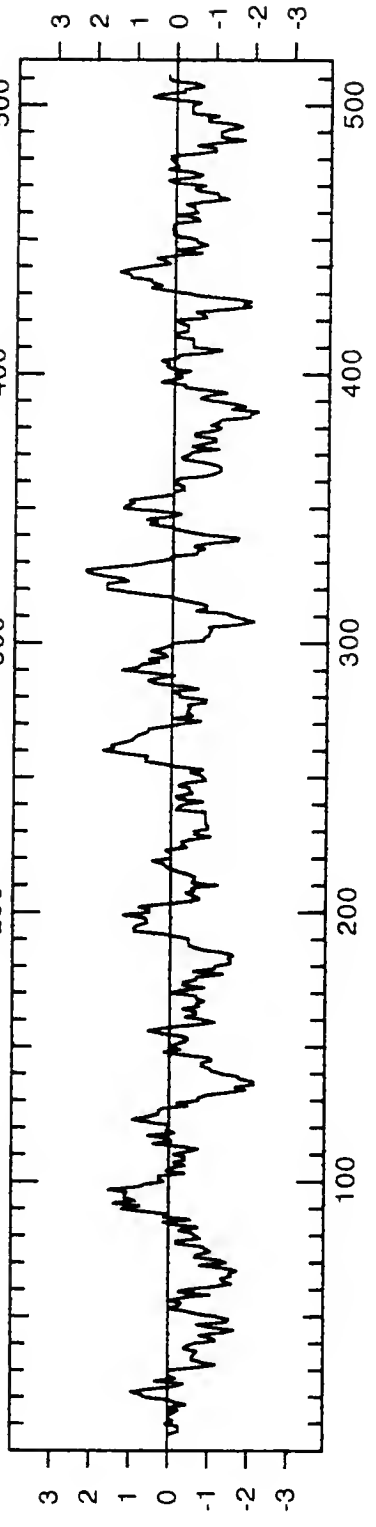

Fig. 2-8 Hydrophobicity plot of the NIb protein sequence of DsMV-Ch1. The horizontal scale indicates amino acid residues in the NIb protein. The hydrophobicity (vertical) scale is that of Kyte and Doolittle (1982), with hydrophobic amino acids above the midline and hydrophilic amino acids below the midline.

Table 2-5. Percent nucleotide identity of CP genes of two DsMV isolates and 14 other potyviruses

Virus[1]	Percent similarity[2]														
	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16
1. DsMV-Ch1	84	68	67	64	64	63	62	60	58	58	58	57	57	56	55
2. DsMV-LA		68	67	65	62	64	60	60	57	58	57	58	54	54	57
3. BCMV			72	71	71	70	58	60	58	59	58	59	57	60	59
4. SbMV				73	80	67	58	59	58	60	58	56	58	61	58
5. PStV					68	67	58	61	57	57	59	58	58	58	57
6. WMV-2						65	60	59	58	59	60	55	55	59	57
7. ZYMV							60	61	57	59	58	57	55	56	59
8. PVY-N								63	62	61	61	56	58	61	61
9. TEV									61	61	62	59	59	63	62
10. PSbMV										59	60	56	59	60	61
11. PRSV-P											59	57	58	60	60
12. TuMV												57	57	56	61
13. TVMV													56	57	59
14. SCMV														55	55
15. BYMV															57
16. PPV															

[1] Name and abbreviations of the viruses used in table: BCMV, bean common mosaic virus; BYMV, bean yellow mosaic virus; DsMV-Ch1, dasheen mosaic virus Ch1 isolate; DsMV-LA, dasheen mosaic virus LA isolate; PPV, plum pox virus; PRSV-P, papaya ringspot virus type P; PSbMV, pea seed-borne mosaic virus; PStV, peanut stripe virus; PVY-N, N strain of potato virus Y; SbMV, soybean mosaic virus; SCMV, sugarcane mosaic virus; TEV, tobacco etch virus; TuMV, turnip mosaic virus; TVMV, tobacco vein mottling virus; WMV-2, watermelon mosaic virus 2; ZYMV, zucchini yellow mosaic virus.

[2] Percent similarity of CP genes among the DsMV-Ch1 and other potyviruses was obtained by Pileup in the GCG program package.

nucleotide level, and from 67% to 82% at the amino acid level (Table 2-4). Individual comparisons showed that potyviruses such as BCMV and SbMV are the most closely related to DsMV among those potyviruses for which the sequences are known. A comparison of the coat protein of DsMV-Ch1 with that of DsMV-LA (Pappu et al., 1994a) revealed a similarity of 84% at the nucleotide level and 92% at the amino acid level, indicating that many of the nucleotide changes observed were silent. Diversity in the coat proteins of the DsMV-Ch1 and other potyviruses occurred predominantly in sequence and length at the N-terminal regions (Shukla et al., 1994). The conserved property of the CPs among different virus strains has been used to classify the potyviruses and their strains (Shukla et al., 1988). Two sorts of phylogenetic trees were obtained by alignment of the coat proteins of the Ch1 and LA isolates of DsMV and either 8 (for exhaustive search) or 14 (for bootstrap search) other potyviruses. The exhaustive search generated a tree showing that both DsMV isolates were closely related to those in the BCMV subgroup (Fig. 2-9); however, in the bootstrap 50% majority-rule consensus tree (Fig. 2-10), the two DsMV isolates were clustered together, and distinct from other potyviruses.

At the N-terminal region of the DsMV-Ch1 CP, there was a DAR triplet at position +5 to +7 in relation to the cleavage site. A single mutation from G to A at the 1986 nucleotide changed the amino residue from a nonpolar glycine to a basic arginine. To confirm this was a true point mutation, ten different clones covering the CP gene were sequenced, and all of them had this point mutation.

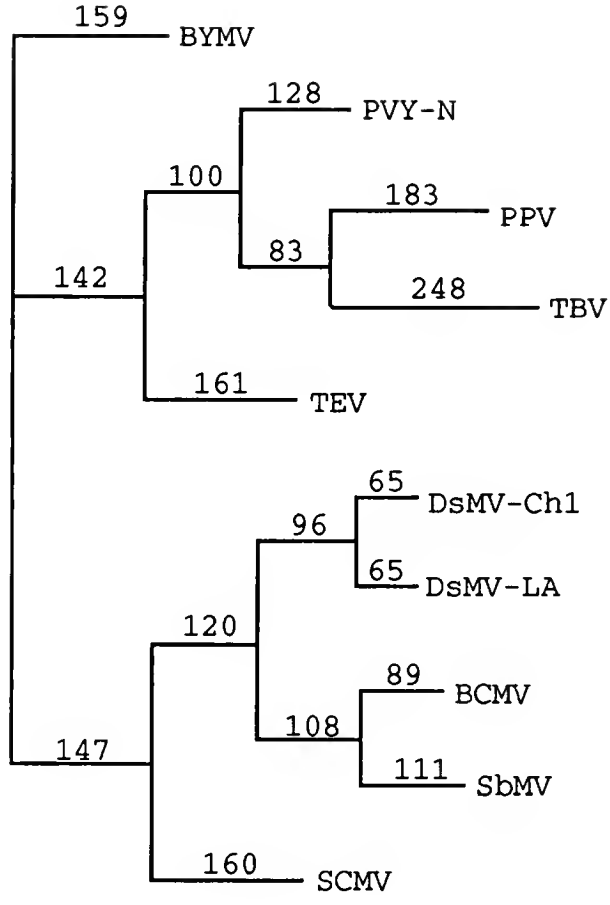

Fig. 2-9. Phylogenetic tree obtained from the alignment of coat proteins between DsMV isolates and 8 other potyviruses using the PAUP program. The tree is the exhaustive consensus tree. The number above a given branch refers to branch length. Vertical distances are arbitrary, and horizontal distances reflect number of amino acid differences between branch nodes.

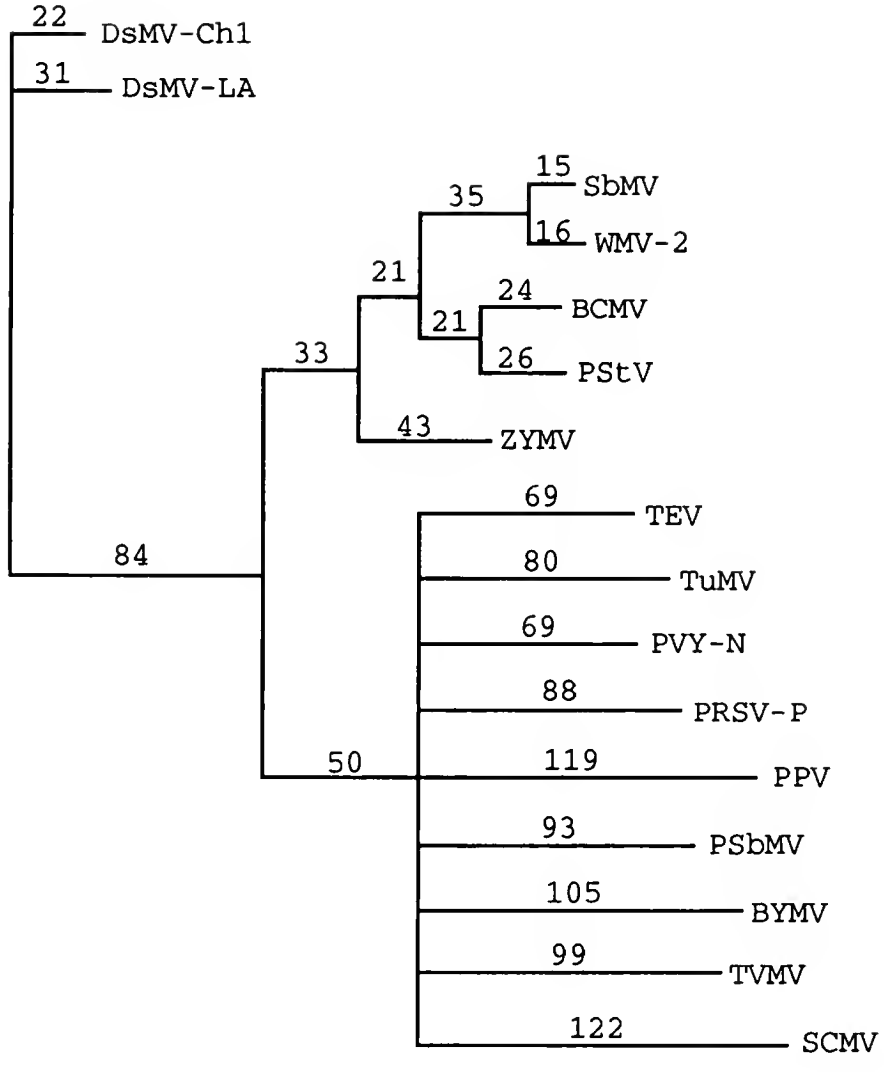

Fig. 2-10. Phylogenetic tree obtained from the alignment of coat proteins between DsMV isolates and 14 other potyviruses using the PAUP program. The tree is the bootstrap 50% majority-rule consensus tree. The number below a given branch refers to branch length. Vertical distances are arbitrary, and horizontal distances reflect number of amino acid differences between branch nodes.

The 3'-NCR of DsMV showed less than 40% homology with those of other potyviruses, but higher than the 84% among Ch1 and LA isolates (Table 2-4). A search for possible secondary structure revealed only short stretches of potentially unstable base pairing.

Aphid Transmission

All three caladium isolates, DsMV-Ch1, -Ch2, and -Ch3 were determined to be transmitted by aphids from their original hosts to *P. selloum* seedlings. Three out of six *P. selloum* plants became infected after aphid inoculation with the Ch1 isolate, two out of six plants with Ch2, and one out of six plants with Ch3, as indicated by symptom expression and positive reactions in I-ELISA.

Expression of the CP and the NIb Genes in *E. coli*

To express DsMV CP as an intact protein, a 1.2 kb DNA fragment was obtained by PCR using two viral specific primers, EH232 and EH234, and subcloned into a pETh-3 expression vector to yield pETh-3-CP. Large quantities of the insoluble DsMV CP were expressed by the pETh-3-CP recombinant (Fig. 2-11). The expressed CP protein was about 39 kDa, which contained a 15-amino-acid residue as a fusion protein. Purification of the expressed protein was facilitated by its insolubility. The protein was partially purified from cell lysates by several cycles of centrifugation and washing the pellets. Further purification was accomplished by preparative SDS-PAGE and electroelution.

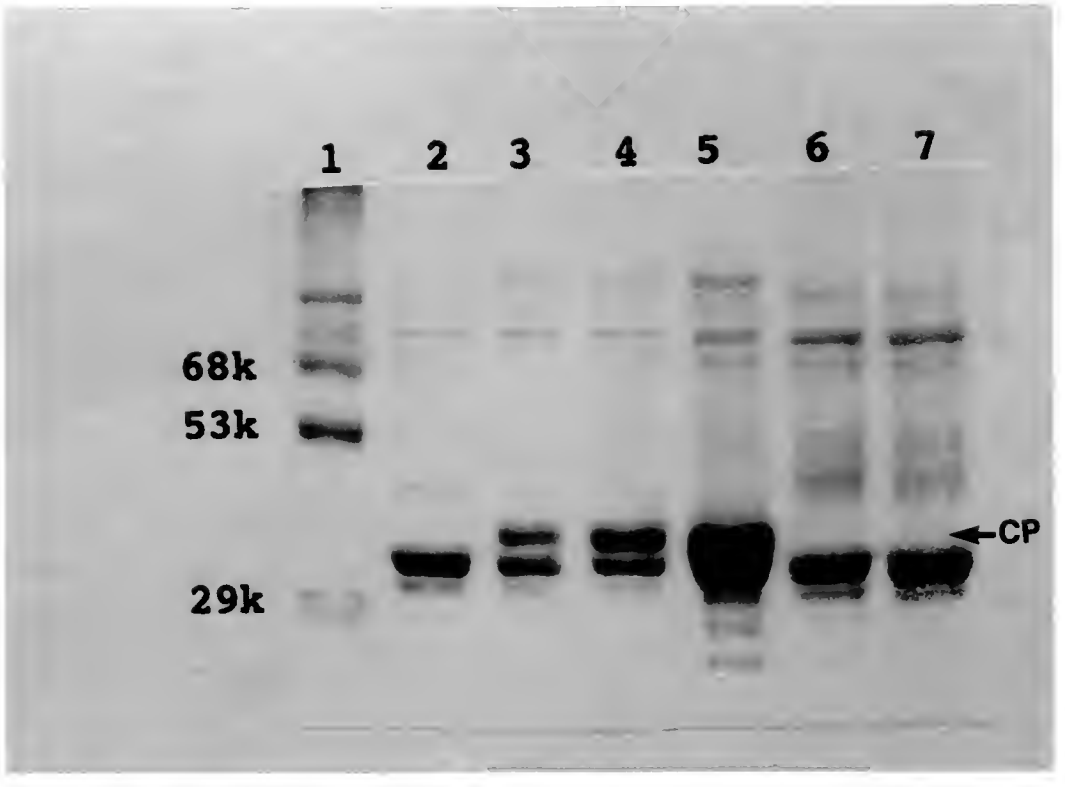

Fig. 2-11. Analysis of the DsMV pETh-3-CP expressed in *E. coli*: Lane 1, protein standards: bovine serum albumin (68 kDa), glutamate dehydrogenase (53 kDa), carbonic anhydrase (29 kDa); lanes 2-5, *E. coli* BL21DE3pLysS with pETh-3-CP, 0, 1, 3, and 5 hr after inducing by IPTG, respectively; Lane 6-7, *E. coli* BL21DE3pLysS with pETh-3 vector, 0 and 5 hr after inducing by IPTG. Partially purified proteins were obtained by centrifuged cell lysates of cultures. Protein samples were analyzed by 10% sodium dodecyl sulfate polyacrylamide gel electrophoresis followed by Coomassie staining. The expressed coat protein of 39 kDa is indicated by the arrow.

The expressed protein reacted with DsMV-FL polyclonal antiserum and with PTY 1 monoclonal antiserum. When comparing it to its homologous state in Western blotting, the expressed DsMV-Ch1 CP was smaller in size than that of the native one (44 kDa) (Fig. 2-12).

The expected DNA fragments of both the intact and truncated NIb genes were obtained by PCR using pDCP12 as the template. The correct clones were confirmed by restricted enzyme digestions and sequencing. However, induction of these clones was unsuccessful in both BL21DE3pLYsS and BL21DE3pLYsE. The latter carries a CE6 plasmid (data not shown). The growth of bacterial cells slowed following induction with IPTG, and no expression was detected.

Application of DsMV Antiserum against Expressed Coat Protein

The expressed DsMV coat protein was a good immunogen and the antiserum prepared to it compared favorably with DsMV-FL antiserum (Table 2-6). The antiserum obtained in the second bleeding reacted with DsMV from infected plants in immunodiffusion tests (data not shown). It also reacted in I-ELISA and Western blotting without any discernible background. Both the antiserum to the expressed CP and the antiserum to the virion reacted with five DsMV isolates, namely Ch1, Ch2, Ch3, Ce, and Xc, as well as ZYMV, PRSV-P, PRSV-W and WMV-2, but not with an isolate of tulip breaking virus from *Lilium*. Neither antiserum reacted with squash mosaic comovirus or with the healthy controls.

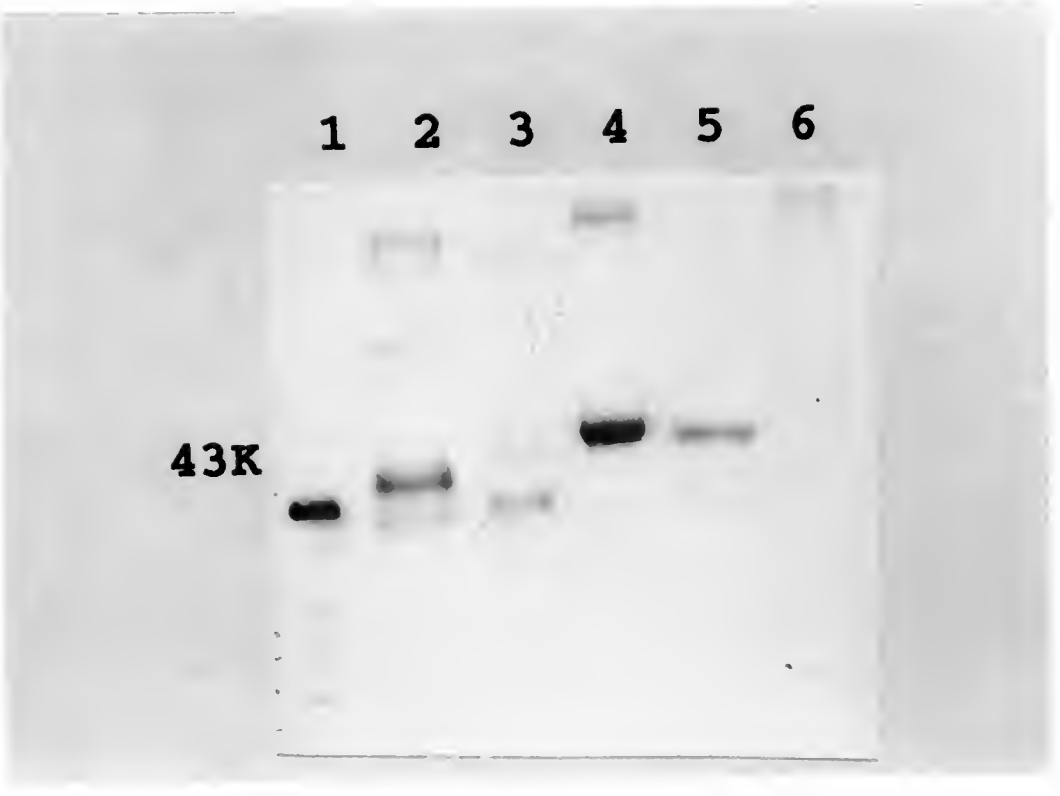

Fig. 2-12. Western blotting analysis of the expressed CP and native CP of DsMV: lane 1, expressed CP; lane 2, extract from DsMV-infected *Caladium hortulanum* 'Candidum' (Ch1); lane 3, extract from DsMV-infected *Colocasia esculenta*; lanes 4-5, extracts from DsMV-infected *Zantedeschia aethiopica* (Za); lane 6, healthy *Philodendron selloum*. Proteins were electrophoresed in a 10% sodium dodecyl sulfate polyacrylamide gel. After electrophoresis, the proteins were blotted onto a nitrocellulose membrane and reacted with DsMV-FL antiserum, and then detected with phosphatase-conjugated goat anti-rabbit antibodies.

Table 2-6. Comparison of the DsMV-FL antiserum and the expressed coat protein antiserum in I-ELISA[1]

Antigen[2]	DsMV-FL AS[3] 1:1000	Expressed CP AS[4] 1:10000
DsMV-Ch1	0.467	0.519
DsMV-Ch2	0.542	0.384
DsMV-Ch3	0.576	0.975
DsMV-Ce1	0.439	0.318
DsMV-X	0.345	0.285
Healthy caladium	0.000	0.002
TBV	0.000	0.001
Healthy lily	0.000	0.003
ZYMV	0.961	0.294
WMV-2	0.720	0.398
PRSV-W	0.431	0.135
SqMV	0.000	0.003
Healthy pumpkin	0.000	0.016

[1] The antisera were compared using I-ELISA as described in the text. The absorbancies (A405) represent the mean values of at least four wells.

[2] Name and abbreviations of the viruses used in test: DsMV, several isolates of dasheen mosaic virus; PRSV-W, papaya ringspot virus type W; PRSV-P, papaya ringspot virus type P; TBV, tulip breaking virus infecting lily; WMV-2, watermelon mosaic virus 2; ZYMV, zucchini yellow mosaic virus; SqMV, squash mosaic comovirus.

[3] Antiserum (rabbit No. 7?) against purified virions of DsMV Florida taro isolate (Abo El-Nil et al., 1977).

[4] Antiserum (rabbit No. 1210) against *E. coli* expressed coat protein of DsMV-Ch1 from caladium isolate.

Discussion

Dasheen mosaic virus antiserum of high quality has only been successfully obtained three times, using viral preparations purified from infected plants (Abo El-Nil et al., 1977; Kositratana, 1985; Shimoyama et al., 1992). Most other workers either failed to purify the virus (Hakkaart & Waterreus, 1975; Samyn & Welvaert, 1977) or could not eliminate host contaminants that interfered with serological tests (Rodoni & Moran, 1988). During the initial experiments in this study, several different virus purification methods, including one described by Abo El-Nil et al. (1977) were tried, also without much success. The isopycnic methods using either phosphate or HEPES buffers were less efficient in preventing irreversible virus aggregation during the extraction process and in reversing virus aggregation during PEG precipitation. Precipitated virions thus were not resuspended and were lost during subsequent low speed centrifugation. In this study, ultracentrifugation using phosphate buffer was used successfully to purify DsMV in conjunction with cesium sulfate density gradient centrifugation.

Philodendron selloum was the only host from which the virus was purified. The virus could only be purified from the first two symptomatic leaves of inoculated *P. selloum* seedlings, however, because the virus titers in the subsequently formed leaves dropped considerably. The yields of DsMV produced in this study were 4-8 mg/100 g of tissue. We were unsuccessful in purifying DsMV from other hosts (caladium, calla lily, cocoyam and taro), despite the high virus titers in these hosts based on the detection of numerous virus particles in negatively stained leaf extracts. This lack of success can be attributed to the highly viscous

nature of leaf extracts of these hosts, which as in other reports (Hakkaart & Waterreus, 1975; Samyn & Welvaert, 1977), interfered with purification. However, it has been reported that DsMV, konjak mosaic and CMV were purified from *Amorphophallus konjak* (Shimoyama et al., 1992). Also, DsMV and an unidentified isometric virus were purified from *Pinellia* (Chen, personal communication).

The viral RNA isolated by the phenol/chloroform extraction yielded more intact DsMV RNA than the sucrose gradient method, which is an important factor to consider when establishing a cDNA library for a virus.

The sequence data of the 3'-terminal region of DsMV-Ch1 support that it is a distinct member of the genus *Potyvirus* in the family *Potyviridae* since the similarities of the virus with other potyviruses were 72 to 85% with the NIb proteins, 67-82% with the coat proteins, and less than 40% with the 3'-NCRs, respectively (Table 2-4). Comparison of NIb proteins and coat proteins of DsMV and 14 other potyviruses revealed a close relationship of DsMV with those in the BCMV subgroup. The phylogenetic trees obtained by alignment of the coat proteins using two exhaustive and bootstrap searches were not the same, however, indicating that differences exist between different search programs. It is interesting to note that all potyviruses except DsMV and those in the BCMV subgroup were clustered together in one group, on the basis of the NIb proteins and the coat proteins (Fig. 2-7 & 2-10, respectively).

Two proteolytic cleavage sites at the C-terminal region of the DsMV-Ch1 were identified. The protease responsible for the cleavage at these sites is the NIa protease. Comparison of determined and predicted cleavage sites in the C-terminal halves of the potyviral

polyproteins revealed that the NIA protein cleaves at the Q/A, Q/G, Q/S, Q/T, Q/V, or Q/E dipeptide sequences (Dougherty & Carrington, 1988, Shukla et al., 1994). Further comparison of the potyviral cleavage sites revealed a conserved sequence VXXQ/A (G, S, E) around the NIa protease cleavage sites. Based on these rules, two putative cleavage sites were identified in the sequenced region of DsMV. The sequence VXLQ/G was found around the cleavage site of the NIa and the NIb proteins, and the sequence VXLQ/A was found around the cleavage site of the NIb and the coat proteins. This conserved cleavage sequence has also been found in SbMV (Jayaram et al., 1992), BCMV isolates (Khan et al., 1993), and PStV (Gunasinghe et al., 1994), suggesting a close relationship among these viral proteases.

Comparison of NIb proteins of different potyviruses have revealed that this protein is the most conserved of the individual potyviral proteins (Shukla et al., 1994). The DSMV-Ch1 NIb protein is 83-85% similar to the analogous proteins of the members in the BCMV subgroup, and 72-76% similar to the other potyviruses compared (Table 2-4). The NIb protein of potyviruses contains the conserved motif SGXXXTXXXNT-18-37aa-GDD, which is conserved in both animal and plant positive-stranded viral RNA-dependent RNA polymerase (Kamer & Argos, 1984). This motif was present in the NIb protein of DsMV as SGQPSTVVDNT-30aa-GDD in a position analogous to that of other sequenced potyviruses when they were aligned according to the sequenced homology. A second consensus motif, YCDADGS, which is also believed to be involved in the putative polymerase activity (Allison et al., 1986; Domier et al., 1986) was present in the NIb protein of DsMV as YCHADGS. When compared with other potyviruses, the motif YCHADGS is present in the NIb proteins in

the members of the BCMV subgroup, while the motif YCDADGS are present in all the other potyviruses compared.

The CP sequences of potyviruses are highly conserved throughout most of the sequence but diverge in sequence and length at the N-terminal region. The CP sequence of DsMV-Ch1 is approximately 80% similar to those of the BCMV subgroup and ranged from 67 to 79% similar to those of other potyviruses compared. The DsMV isolates displayed higher levels of homology in the CP sequences (92-96%). From phylogenetic analysis using PAUP, it is clear that DsMV is a distinct virus, albeit relatively close to the potyviruses in the BCMV subgroup.

According to the CP sequence, DsMV-Ch1 was not expected to be aphid-transmissible, since it did not have the DAG sequence in the N-terminus portion of the CP as is typical for aphid-transmissible potyviruses (Harrison & Robinson, 1988). Instead, it had the DAR at the N-terminal region of the coat protein. However, when the aphid transmission tests were conducted using DsMV-Ch1 from the infected caladium as virus source, this isolate proved to be aphid transmissible. Furthermore, when the same isolate was cloned by RT-PCR from the original host (caladium cultivar `Candidum') and sequenced, the triplet DAG rather than DAR was present. Kositratana reported (1985) that a California DsMV isolate from Chinese evergreen plants was not aphid-transmissible from infected *P. selloum* plants to healthy *P. selloum* seedlings, which might also resulted from the mutation at the DAG triplet. It is probable that mutation at this triplet was frequent, due to the propagation of a variant in *P.*

selloum seedlings. This variant might have been present in the original DsMV infected caladium plants, or it could have been the result of a single point mutation (GGG to AGG).

The antiserum against the *E. coli* expressed CP of DsMV-Ch1 reacted with DsMV isolates and other potyviruses tested in a manner similar to that of the DsMV-FL antiserum obtained by Abo El-Nil et al. (1977). The DsMV antisera not only reacted with its homologous isolates, but also with most potyviruses tested, which indicated that the close relationship of DsMV with these other potyviruses. The expression of the CP gene *in vitro* provided a suitable alternative for obtaining immunogen for antiserum production to this virus, which thus far has been difficult by conventional means.

CHAPTER 3
VARIABILITY OF COAT PROTEINS AMONG ISOLATES OF DASHEEN MOSAIC VIRUS

Introduction

Many cultivated aroids have become ubiquitously infected with dasheen mosaic virus throughout the world, since they are exclusively propagated by vegetative means and thus can harbor the virus indefinitely (Zettler & Hartman, 1977). Although DsMV has been reported from many countries, the relationships between isolates have not yet been studied extensively. A severe DsMV isolate of taro has been reported in French Polynesia (Jackson, 1982). Biological and serological differences between isolates were noticed in infected taro from Egypt and Florida (Abo El-Nil et al., 1977), and between Florida and Fiji isolates of taro (Abo EL-Nil et al., 1977). The serological difference between a California isolate from Chinese evergreen and two isolates from taro, Florida isolate and Fiji isolate (Kositratana, 1985). It has been reported that the estimated molecular weights of DsMV coat protein varied among isolates from different hosts (Li et al., 1992; Pappu et al., 1994b), and this variability could be associated with diversity on the N-terminal region of the CPs (Pappu et al., 1994b).

The accumulating body of knowledge of coat protein sequences has been used by various authors to differentiate potyviruses and their strains. Comparisons of the growing number of potyviruses sequenced revealed that distinct potyviruses show only 38-71%

sequence homology in their coat proteins, whereas this homology is greater than 90% among strains belonging to the same virus (Shukla & Ward, 1988, 1989). Furthermore, the 3' non-coding regions (3'-NCRs) of different potyviruses also display a high degree of sequence variation (similarity of 39-45% only), whereas sequences are highly conserved between virus strains (similarities of 83% and more). Recently, the coat protein and the 3'-NCR sequences of four DsMV isolates (Pappu et al., 1994a; Li et al., 1994; this study) are available to be compared.

Materials and Methods

Antigens

Florida isolates of DsMV used in this study were DsMV-Ce from taro (*Colocasia esculenta*), and DsMV-Ch1, -Ch2, -Ch3 from three cultivars of caladium (*Caladium hortulanum*), `Candidum', `Carolyn Whorton' and `Frieda Hemple', respectively. The calla lily (*Zantedeschia aethiopica*) isolate, DsMV-Za, was from California; and the cocoyam (*Xanthosoma caracu*) isolate, DsMV-Xc, was from Puerto Rico. These isolates were maintained in their original hosts throughout this investigation. The isolates were also maintained in mechanically inoculated *P. selloum* seedlings throughout the study. Symptoms of *P. selloum* usually appeared 2 weeks after inoculation, and the first symptomatic leaf formed after inoculation was routinely tested.

Antigens of PRSV-W, PStV, PepMoV, PVY, TEV, WMV-2 and ZYMV were provided by D. E. Purcifull (Department of Plant Pathology, University of Florida,

Gainesville). TVMV was from T. P. Pirone (Department of Plant Pathology, University of Kentucky, Lexington). A Puerto Rican passionfruit potyvirus (Bird et al., 1991) was provided by A. C. Monllor (Department of Crop Protection, University of Puerto Rico, Rio Piedras). Also tested in this investigation were antigens of a gladiolus isolate of BYMV (Nagel et al., 1983), bidens mottle collected from *Bidens pilosa* in the campus of the University of Florida (Gainesville), and two strains of PMoV from peanut and bambarra groundnut (Li et al., 1991).

Antisera

The purified DsMV-FL IgG of Abo El-Nil et al. (1977) was routinely used in this study. Antisera of BlCMV, PRSV-W, PMoV, PStV, PVY, TEV, WMV-2 and ZYMV were provided by D. E. Purcifull. Antisera to the Puerto Rican passionfruit potyvirus was provided by A. C. Monllor. The PTY 1 potyvirus group cross reactive monoclonal antiserum (PTY 1) was purchased from Agdia, Inc. (Elkhart, IN).

Propagations and Analysis of DsMV Isolates

Each DsMV isolate in its original host was maintained in a greenhouse. The leaf samples were collected from infected plants and tested by Western blotting as described in Chapter 4.

Six DsMV isolates, -Ch1, -Ch2, -Ch3, -Ce1, -Xc and -Za were used to inoculate *Philodendron selloum* seedlings at the 7-8 leaf stage in the spring months of 1991. Each

isolate was then serially transferred at least twice to additional plants of *P. selloum* seedlings. The first symptomatic leaves were collected from the infected plants of each passage, and prepared for Western blotting.

Each isolate was also used to inoculate tissue culture-derived, virus-free plants of taro, cocoyam, and the caladium cultivars `Candidum', `Carolyn Whorton', `Frieda Hemple' and `Rosebud'. The leaf tissues of the infected plants were prepared for and tested by Western blotting.

Cloning and Nucleotide Sequencing of PCR-amplified CP Genes

The CP genes of the DsMV isolates Ch1a and Ch2 were obtained by RT-PCR as described in Chapter 2. The DsMV-Ch1a was from caladium cultivar `Candidum' which was the same host from which DsMV-Ch1 was isolated, and -Ch2 was from caladium cultivar `Carolyn Whorton'. After electrophoresis, the desired amplified CP fragments were isolated by using Prep-A-Gene Kit (Bio-Rad Laboratories, Hercules, CA), ligated into pGEM-T vector (Promega Co., Madison, WI), and transformed into competent *Escherichia coli* DH5α cells. The recombinant colonies were screened by the blue-white color reaction. The plasmid preparations from the selected clones were then sequenced by the termination method (Untited States Biochemical, Cleveland, OH) using T7, SP6 vector primers and DsMV CP-specific internal primers. The nucleotide sequences were determined for both strands.

Sequence and Comparison of the CP and the 3'-NCR

The nucleotide sequences of the coat protein genes and the 3' non-coding regions of three caladium isolates, Ch1, Ch1a, Ch2, and the two taro isolates, LA and TEN, sequenced by Pappu et al. (1994b) were compiled, and analyzed. The Ch1a isolate was cloned from the original host of the DsMV-Ch1, caladium cultivar 'Candidum' by RT-PCR. The level of the sequence relatedness was compared using Pileup available in the GCG program package from the University of Wisconsin (Devereux et al., 1984).

Results

Symptoms in *P. selloum*

Each of six isolates tested induced systematic vein chlorosis in the first and/or second leaves of *P. selloum* after mechanical inoculation. However, the isolates DsMV-Ce, -Ch1, and -Ch2 induced more severe symptoms in *P. selloum* than those of the Ch3, Xc and Za isolates. The mild isolates induced chlorotic spots in the first symptomatic leaves, whereas severe isolates induced pronounced stunting. Similar symptom differences among DsMV isolates in *P. selloum* have been described by Abo El-Nil et al. (1977) and Wisler et al. (1978).

The Coat Proteins of DsMV in Their Original Hosts

The estimated CP molecular weight (MW) of each of six DsMV isolates from their original hosts varied (Fig. 3-1). In Western blots using DsMV antiserum, the respective highest CP MW values for the isolates Ch1, Ch2, Ch3, Ce, Xc, and Za were 44, 46, 38, 44, 47 and 43 kDa, whereas values for the other eleven potyviruses tested were much lower, ranging from 31 kDa for TEV to 36 kDa for the Puerto Rican passionfruit virus (Fig. 3-2). Similar results were noticed when polyclonal antisera of BlCMV, PRSV-W, PMoV, PStV, TEV, WMV-2, ZYMV, and the Puerto Rican passionfruit potyvirus, were tested. In reciprocal tests with DsMV and two PMoV strains from peanut and bambarra groundnut, homologous reactions were much stronger than heterologous ones. Likewise, homologous reactions were stronger than heterologous reactions when two PMoV strains were compared against the other potyviruses (i.e. PRSV-W, PStV, TEV, WMV-2, and the Puerto Rican passionfruit potyvirus).

The Coat Proteins of DsMV Isolates in Other Hosts

The CP MWs corresponding to those noted in their respective original hosts were detected for each of six DsMV isolates infecting *P. selloum*. Each isolate was manually inoculated to plants of *P. selloum*, caladium, cocoyam and taro (Fig. 3-3). Similarly, after two or more serial passages through manually inoculated *P. selloum* seedlings, respective CP MW values remained consistent for each of the DsMV isolates tested (Fig. 3-4).

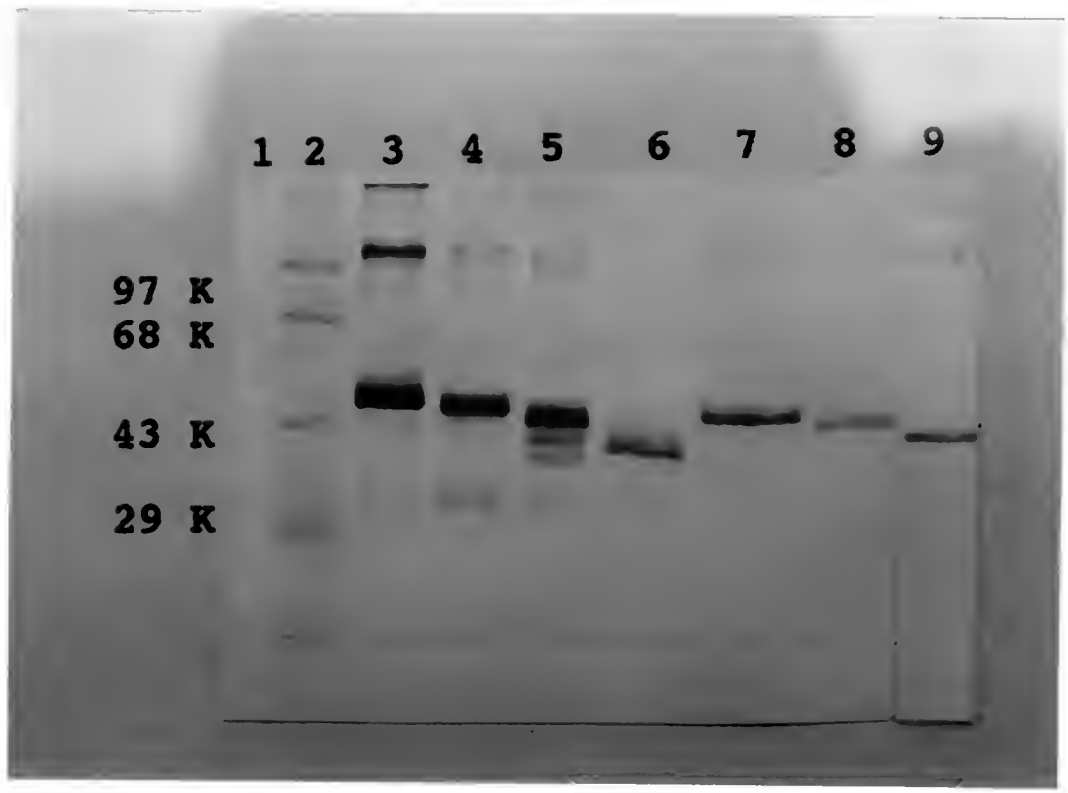

Fig. 3-1. Western blotting analysis of the coat proteins of dasheen mosaic virus (DsMV) isolates from their original hosts: lane 1, healthy *Colocasia esculenta*; lane 2, BRL protein standard; lane 3, *Xanthosoma caracu* (Xc); lane 4, *Colocasia esculenta* (Ce); lane 5, *Caladium hortulanum* 'Carolyn Whorton' (Ch2); lane 6, *C. hortulanum* 'Frieda Hemple' (Ch3); lane 7, *C. hortulanum* 'Candidum' (Ch1); and lane 8, *Zantedeschia aethiopica* (Za). Proteins were electrophoresed in a 10% sodium dodecyl sulfate polyacrylamide gel. After electrophoresis, the proteins were blotted onto a nitrocellulose membrane and reacted with DsMV-FL antiserum, and then detected with phosphatase-conjugated goat anti-rabbit antibodies.

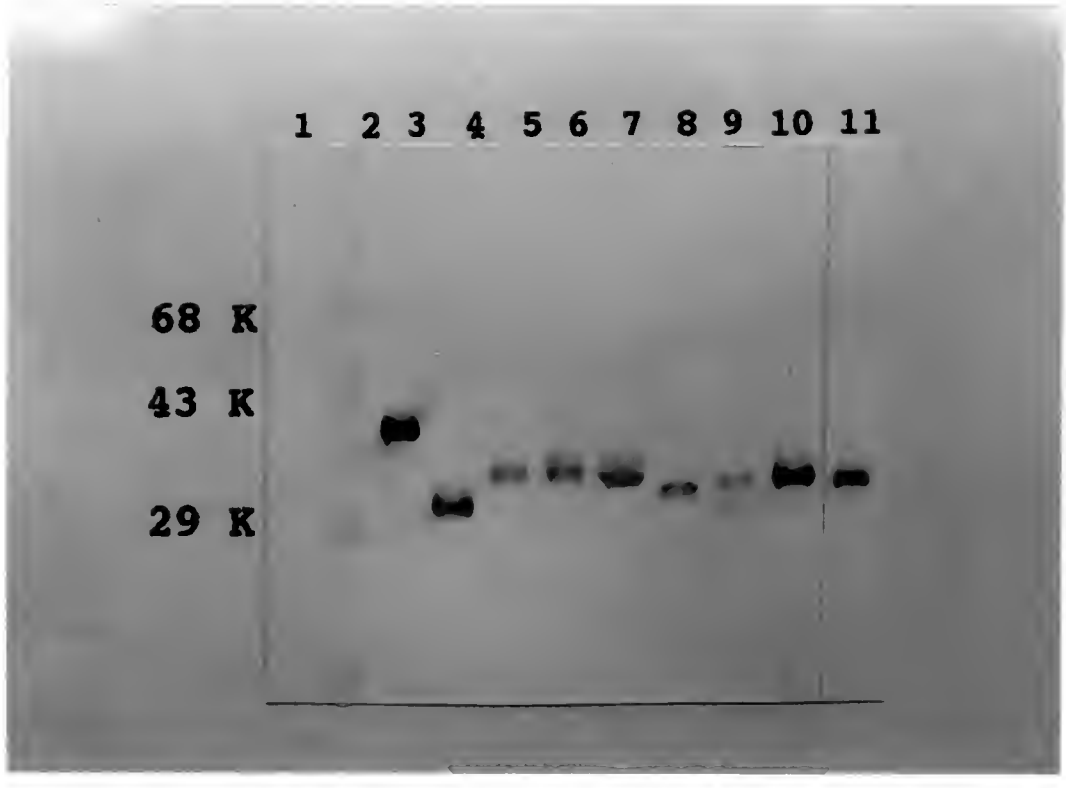

Fig. 3-2. Western blotting analysis of the Ch1 isolate of dasheen mosaic virus (DsMV-Ch1) and other potyviruses: lane 1, healthy *Philodendron selloum*; lane 2, BRL protein standard; lane 3, DsMV-Ch1; lane 4, tobacco etch virus; lane 5, passionfruit potyvirus; lane 6, watermelon mosaic virus 2; lane 7, papaya ringspot virus type W; lane 8, zucchini yellow mosaic virus; lane 9, pepper mottle virus; lane 9, potato virus Y; lane 10, bean yellow mosaic virus (gladiolus strain); lane 11, bidens mottle virus. Proteins were electrophoresed in a 10% sodium dodecyl sulfate polyacrylamide gel. After electrophoresis, the proteins were blotted onto a nitrocellulose membrane and reacted by DsMV-FL antiserum, and then detected with phosphatase-conjugated goat anti-rabbit antibodies.

Fig. 3-3. Western blotting analysis of the Ch1 isolate of dasheen mosaic virus (DsMV-Ch1) transferred to several different hosts: lane 1, BRL protein standard; lane 2, healthy *Philodendron selloum;* lane 3, *Xanthosoma caracu*; lane 4, *Colocasia esculenta*; lane 5, *Caladium hortulanum* 'Carolyn Whorton'; lane 6, *C. hortulanum* 'Candidum'; lane 7, *C. hortulanum* 'Candidum'; lane 8, *C. hortulanum* 'Frieda Hemple'; lane 9, healthy *C. hortulanum* 'Candidum'. Proteins were electrophoresed in a 10% sodium dodecyl sulfate polyacrylamide gel. After electrophoresis, the proteins were blotted onto a nitrocellulose membrane and reacted with DsMV-FL antiserum, and then detected with phosphatase-conjugated goat anti-rabbit antibodies.

Fig. 3-4. Western blotting analysis of four dasheen mosaic virus (DsMV) isolates serially propagated in *Philodendron selloum* seedlings: lane 1, BRL protein marker; lanes 2 and 14, healthy *P. selloum;* lanes 3-5, Ce1 isolate from *Colocasia esculenta*; lanes 6-8, Ch1 isolate from *Caladium hortulanum* 'Candidum'; lanes 9-11, Xc isolate from *Xanthosoma caracu*; and lanes 12-13, Ch2 isolate from *C. hortulanum* 'Carolyn Whorton'. The proteins were electrophoresed in a 10% sodium dodecyl sulfate polyacrylamide gel. After electrophoresis, the proteins were blotted onto a nitrocellulose membrane and detected with DsMV-FL antiserum, and then detected with phosphatase-conjugated goat anti-rabbit antibodies.

Comparison of the Coat Protein Sequences

The alignment of the CP sequences of the caladium isolates, Ch1, Ch1a, Ch2, and the taro isolates, LA and TEN (Pappu et al., 1994b) showed (Fig. 3-5). The amino acid sequences were deduced from the nucleotide sequences. Fig. 3-6 shows the multiple alignment of the predicted amino acid sequences for these DsMV isolates. Sequence homologies of CP genes among these DsMV isolates were 84% to 96% at the nucleotide level, and 92% to 96% at the amino acid level. However, the CP of the LA isolate (330 amino acids) was 15-16 amino acids longer than those of the three other isolates studied: 314 amino acids for isolates Ch1 and Ch2, and 315 amino acids for the TEN isolate (Fig. 3-6). These differences can be attributed to a 12-base addition and a 57-60 base deletion at the 5'-terminal region of the CP gene of the Ch1, Ch2 and TEN isolates. The addition occured between positions + 49 and + 50 and corresponded to amino acid residues of 17 to 20 of the CPs of the Ch1, Ch2 and TEN isolates. The deletion was from +94 (+97 for the isolate TEN) to +154, corresponding to amino acid residues 32 (33 for isolate TEN) to 51 of the LA isolate. The addition and deletion occurred within the unusual threonine and asparagine rich portion of the N-terminal region.

Among isolates Ch1, Ch1a, Ch2 and TEN, the N-terminal region of the coat proteins appeared highly conserved, both in length (76-77 amino acids) and in sequence (similarity 71% to 78%). While the N-terminal region of the LA isolate was larger in size (92 amino acids), its sequence similarity was 71-73%. Furthermore, the CP core and the C-terminal region of the Ch1, Ch2, LA and TEN isolates were all fairly conserved both in

Fig. 3-5. Comparison of nucleotide sequences of the coat protein (CP) gene of dasheen mosaic virus isolates Ch1 and Ch1a (from *Caladium hortulanum* 'Candidum'), Ch2 (from *Caladium hortulanum* 'Carolyn Whorton'), LA and TEN (from *Colocasia esculenta*). Nucleotide coding for the N-terminal regions of the CPs are underlined, and the stop codons are shown by asterisks.

```
          1                                                   48
DsMV-LA   GCTGATGACA CAGTTGATGC AGGGAATCAG AACAATACCA ATAAAACA..
DsMV-TEN  GCAGATGACA CAGTTGATGC AGGGAACCAG AATAATACTA ATAAAACAAC
DsMV-Ch1  GCTGATGATA CAGTTGATGC AAGGAAAAAC AACAATACTA CAAAAACAAC
DsMV-Ch1a GCTGATGATA CAGTTGATGC AGGGAAAAAC AACAACACTA CAAAAACAAC
DsMV-Ch2  GCTGATGATA CAGTTGATGC AGGGAAGAAT AGCAAAAACA CAAAAACAAC
```

N-terminus

```
49                                                    88
.......... ACCCCTGCAG CTGGTGGTGG TAACAACACA AATACCAACA
CGAAACAAAG ACTCCTGCAG CAAGTGGTGG TAACAACACA AAT.......
TGAAACAAAA ACACCTGCAA CGGGTGGTGG GAACAACACA AAC.......
TGAAACAAAA ACACCTGCAA CGGGCGATGG GAACAACACA AAC.......
TGAAACAAAA ACTCCTGCAT CGGGTCGTGG CAACAACACC AAC

89                                                   138
CCAATACTGG TAACAACACA AACACCAATA CCAGTACTGG TAACAATACA
.......... .......... .......... .......... ..........
.......... .......... .......... .......... ..........
.......... .......... .......... .......... ..........
.......... .......... .......... .......... ..........

139                                                  188
AACACCAACA CCAACACTAA TACCAACACA ACCAATAATA ATCCTCCACC
AATACTCCAC CACCACCCGC AAACAACACA ACTAATAACA ATCCTCCACC
...AACAACA CGCCACCTGT AGATAACACA ACCAACAATA ATCCTCCACC
...GACAACA CGCCACCTGT AGGTAACACA ACCAACAATA ATCCTCCACC
AACAGCACTA CACCACCTGC AAATAACAAC ACAAACAACA ATCCTCCACC

189                                                  238
GCCACCACCG GCGGCACCAA AAGCTTCAGA GACGCCAGCA AACAAGCAGG
GCCACCACCG ACGGCACCAA AGGCGACAGA GACGCCAGCC AACACACAAG
GCCACCACCG GCGGTTACAA AGGCAACAGA GATACCCGCC AATAAGCAAG
ACCACCACCG GCGGTTCCAA AGGTAGCAGA GATACCCGCC AATAAGCAAG
ACCGCCACCA GGCGCGCCAA AAGCAACAGA GACGCCGGCT AACAAACAAG

239                                                  288
TAGTCCCCAC AACAAGTGAT AAAGGTAAGG AGATTGTTAA AGATGTCAAT
TAGTCCCAAC GGCAAGTGGG AAAGGTAAGG AAGTTGTTAA AGATGTCAAC
TGGTCCCAGC AGCAAGTGAG AAAGGTAAGG AAATTGTGAA AGATGTTAAC
TGGTCCCAGC AGCAAGTGAG AAAGGTAAGG AAATTGTGAA AGATGTTAAC
TCGTCCCCCA AACAAATGAG AAACGGAAGG AAGTGGTCAA AGATGTCAAC
```

Fig. 3-5--Continued

```
          289                                                  338
DsMV-LA   GCTGGCACAA GTGGAACATA CTCTGTACCT CGGTTAAACA AAATCACAAA
DsMV-TEN  GCTGGCACAA GTGGCACATA TTCTGTACCT CGATTGAATA AAATCACAAA
DsMV-Ch1  GCTGGCACTA GTGGCACACA CTCTGTACCT CGGTTGAATA GAATCACAAA
DsMV-Ch1a GCTGGCACTA GTGGCACATA CTCTGTACCT CGGTTGAATA AAATCACAAA
DsMV-Ch2  GCTGGCACCA GTGGCACGTA CTCTGTACCT CGATTAAACA GAATCACACA

          339                                                  388
          TAAAATGAAC TTGCCTTTAG TCAAAGGCAA GTGCATTTTA AATTTAAATC
          CAAGATGAAT TTACCTTTAG TTAAAGGTAA GTGCATTTTA AATTTAAACC
          CAAAATGAAT TTACCTTTAG TTAAAGGTAA ATGCATTCTA AATTTGAATC
          CAAAACGAAT TTACCTTTAG TTAAAGGTAA ATGCATTTTA AATTTGAATC
          CAAAATGAAC TTACCTTTAG TTAAAGGTAA ATGCATTTTG CATTTAAATC

          389                                                  438
          ATTTAATCGA GCACAAACCT GAGCAGCGTG ACATATTCAA CACCAGAGCC
          ATTTAATCGA GTACAAACCC GAACAGCGTG ACATCTTCAA TACCAGAGCC
          ATTTAATCGA GTACAAGCCA GAACAGCGCG ACATATTCAA TACCAGAGCC
          ATTTAATCGA GTACAAGCCA GAACAGCGTG ACATATTCAA TACCAGAGCC
          ACTTGATCGA GTACAAACCA GAACAGCGAG ACATCTTCAA TACCAGAGCC

          439                                                  488
          ACCCACACAC AGTTTGAGGT CTGGTACAAT GCTGTCAAGA GGGAATATGA
          ACCCACACAC AATTCGAGGT ATGGTACAAC GCCGTTAAGA GGGAGTACGA
          ACCCACACTC AATTTGAAGT CTGGTACAAT GCTGTCAAGA GAGAATACGA
          ACCCACACTC AATTTGAAGT CTGGTACAAT GCTGTCAAGA GAGAATACGA
          ACCCACACAC AATTTGAGGT CTGGTACAAT GCTGTCAAGA GGGAATATGA

          489                                                  538
          GCTCGAGGAT GAGCAGATGC ATATTGTTAT GAATGGTTTC ATGGTTTGGT
          GCTTGAGGAC GAGCAGATGC ACATTGTAAT GAATGGTTTC ATGGTTTGGT
          GCTTGAGGAT GAGCAGATGC ACATAGTTAT GAATGGTTTT ATGGTTTGGT
          GCTTGAGGAT GAGCAGATGC ACATAGTTAT GAATGGTTTT ATGGTTTGGT
          GCTCGAGGAT GAGCAGATGC ACATTGTTAT GAACGGTTTC ATGGTTTGGT

          539                                                  588
          GCATCGACAA TGGAACATCA CCTGACATTA ACGGGGCTTG GGTGATGATG
          GCATCGATAA TGGAACATCA CCCGATATCA ACGGGGCTTG GGTGATGATG
          GCATCGATAA TGGAACATCA CCTGATATCA ACGGGGCTTG GGTGATGATG
          GAACATCACC TGATATCAAC GGGGCTTGGG TGATGATGGC ATCGATAGTG
          GCATCGACAA TGGAACTTCA CCCGACATCA ACGGGGCTTG GGTGATGATG
```

Fig. 3-5--Continued

```
          589                                                        638
DsMV-LA   GATGGAAATG ATCAAATTGA ATACCCGTTA AAGCCGATTG TGGAGAATGC
DsMV-TEN  GACGGAAACG ATCAGATTGA ATACCCGTTA AAACCAATTG TGGAAAATGC
DsMV-Ch1  GACGGAAACG ATCAAATTGA ATACCCGTTG AAGCCAATTG TTGAAAATGC
DsMV-Ch1a GATGGAAGTG ATCAAATTGA ATACCCGTTA AAACCAATTG TCGAAAATGC
DsMV-Ch2  GACGGAAATG ATCAAATTGA ATACCCGTTA AAGCCGATCG TGGAAAATGC

          639                                                        688
          AAAACCCACC TTGCGTCAGA TAATGCATCA CTTTTCTGAC GCAGCAGAGG
          AAAACCCACC TTGCGTCAGA TAATGCATCA CTTTTCTGAC GCAGCAGAGG
          AAAACCAACC TTGCGTCAGA TAATGCATCA CTTTTCTGAC GCAGCAGAGG
          AAAACCAACT TTGCGTCAGA TTATGCATCA CTTTTCTGAC GCAGCAGAGG
          AAGACCCACC TTGCGTCAGA TAATGCATCA CTTTTCTGAC GCAGCAGAGG

          689                                                        738
          CTTATATTGA ACTGAGAAAT GCGGAAAAAC CATACATGCC TAGGTATGGT
          CTTATATCGA ACCGAGGAAT GCGGAGAAAC CATACATGCC TAGGTATGGT
          CATACATTGA ACTGAGAAAC GCAGAGAAAC CGTATATGCC TAGATACGGT
          CATACATTGA ATTGAGGAAC GCGGAGAAAC CGTACATGCC TAGGTATGGT
          CGTACATTGA ACTGAGAAAT GCGGAAAAAC CATACATGCC TAGGATAGGT

          739                                                        788
          CTTATTCGCA ACTTACGTGA TGCAAGTCTC GCCCGGTACG CTTTCGACTT
          CTCATTCGCA ATCTACGTGA TGCAAGTCTT GCCCGGTATG CTTTCGACTT
          CTTATTCGCA ATTTACGTGA TGCAAGTCTC GCCCGGTATG CTTTTGACTT
          CTCATCCGCA ACTTACGTGA TGCAAGTCTT GCCCGGTATG CTTTTGACTT
          CTAATTCGCA ACTTACGTGA TGCAAGTCTC GCCCGGTATG CTTTCGACTT

          789                                                        838
          CTATGAAGTT AACTCTAAAA CACCGGTCCG AGCAAGGGAG GCAGTTGCGC
          CTATGAGGTC AATTCTAAAA CACCGGTGCG AGCGAGAGAA GCAGTCGCGC
          TTATGAGGTC AATTCTAAAA CACCGGTGCG AGCAAGAGAA GCAGTTGCGC
          CTATGAGGTC AATTCTAAAA CACCGGTGCG AGCAAGAGAA GCAGTTACGC
          CTATGAAGTC AATTCTAAGA CGCCGGTACG AGCAAGAGAG GCAGTAGCGC

          839                                                        888
          AAATGAAGGC CGCTGCACTT TCTAACGTTA CCACTAGGTT GTTTGGTTTG
          AAATGAAGGC CGCTGCGCTC TCCAACGTTA CCACTACCTT GTTTGGTTTG
          AAATGAAGGC GGCTGCACTC TCTAACGTTA CCACTAGGTT GTTTGGTTTG
          GGATGAAGGC CGCTGCACTC TCTAACGTTA CCACTAGGTT GTTTGGTTTG
          AGATGAAGGC TGCTGCACTC TCTAACGTTA CCACTAGGTT GTTTGGTTTG
```

Fig. 3-5--Continued

```
          889                                                    938
DsMV-LA   GATGGTAACG TTTCGACTTC AAGCGAGAAC ACTGAAAGGC ACACTGCAAA
DsMV-TEN  GATGGTAACG TTTCAACTTC AAGCGAGAAC ACTGAAAGGC ATACTGCAAA
DsMV-Ch1  GATGGTAACG TTTCAACTTC AAGCGAGAAC ACTGAAAGGC ACACTGCAAA
DsMV-Ch1a GATGGTAACG TTTCAACTTC AAGCGAGAAC ACTGAAAGGC ACACTGCAAA
DsMV-Ch2  GATGGTAACG TTTCAACTTC AAGCGAGAAC ACTGGAAAGC ACACTGCAAA

          939                                                    990
                                                                 *
          AGACGTCACA CCCAACATGC ATACATTGCT TGGTGTGGCA TCTCCACAGTAA
          GGATGTCACG CCAAATATGC ACACCTTGCT CGGCGTAGCG CCTCCGCAGTAA
          AGACGTCACA CCAAACATGC ACACTTTACT TGGTGTTTCG TCTCCGCAGTAA
          AGACGTCACA CCAAACATGC ACACTTTACT TGGCGTTTCG TCTCCGCAGTAA
          AGACGTCACA CCAAACATGC ACACTTTACT TGGCGTTTCG TCTCCGCAGTAA
```

Fig. 3-5--Continued

```
          1                                                  46
DsMV-LA   ADDTVDAGNQ NNTNKT.... TRAAGGGNNT NTNTNTGNNT NTNTSTGNNT
DsMV-TEN  ---------- ------TETK ----S----- -......... ..........
DsMV-Ch1  -------RKN ---T--TETK -P-T------ -......... ..........
DsMV-Ch1a ---------- ------TETK -P-T-D---- -......... ..........
DsMV-Ch2  --------KN SKNT--TETK -P-S-R---- N......... ..........

          47                                                  96
          NTNTNTNTNT TNNNPPPPPP AAPKASEIPA NKQVVPTTSD KGKEIVKDVN
          --PP-PAN-- ---------- TA-------- -T-----A-G ---V------
          .-N-PPVD-- ---------- -VT--T---- ------AA-E ----------
          .D-----G-- ---------- ----V----- ---------- ----------
          -ST-PPAN-N ---------- G----T-T-- ------Q-NE -R--V-----

          97                                                 146
          AGTSGTYSVP RLNKITNKMN LPLVKGKCIL NLNHLIEYKP EQRDIFNTRA
          ---------- ---------- ---------- ---------- ----------
          ------H--- ---R------ ---------- ---------- ----------
          ---------- --------T- ---------- ---------- ----------
          ---------- ---R--H--- ---------- H--------- ----------

          147                                                196
          THTQFEVWYN AVKREYELED EQMHIVMNGF MVWCIDNGTS PDINGAWVMM
          ---------- ---------- ---------- ---------- ----------
          ---------- ---------- ---------- ---------- ----------
          ---------- ---------- ---------- ------S--- ----------
          ---------- ---------- ---------- ---------- ----------

          197                                                246
          DGNDQIEYPL KPIVENAKPT LRQIMHHFSD AAEAYIELRN AEKPYMPRYG
          ---------- ---------- ---------- ---------- ----------
          ---------- ---------- ---------- ---------- ----------
          --S------- ---------- ---------- ---------- ----------
          ---------- -------R-- ---------- ---------- --------I-

          247                                                296
          LIRNLRDASL ARYAFDFYEV NSKTPVRARE AVAQMKAAAL SNVTTRLFGL
          ---------- ---------- ---------- ---------- ----------
          ---------- ---------- ---------- ---------- ----------
          ---------- ---------- ---------- --TR------ ----------
          ---------- ---------- ---------- ---------- ----------

          297                                 330
          DGNVSTSSEN TERHTAKDVT PNMHTLLGVS SPQ*
          ---------- ---------- ---------- ---*
          ---------- ---------- ---------- ---*
          ---------- ---------- ---------- ---*
          ---------- -GK------- ---------- ---*
```

Fig. 3-6. Comparison of amino acid sequences of coat proteins of dasheen mosaic virus isolates LA, TEN, Ch1, Ch1a and Ch2. Identical amino acids are shown by dashes and gaps are indicated by dots.

length (237 amino acids) and in sequence (98% at the amino acid level) (Fig. 3-6). The overall sequence similarity of the coat proteins was quite high and comparable to identities observed between strains of a single potyvirus species (Shukla & Ward, 1988; Ward et al., 1992).

The 3'-NCR of the Ch1, Ch2 and LA isolates were all similar in length (246, 243 and 247 nt, respectively). The sequence identity in this region ranged from 79-83% (Fig. 3-7).

Discussion

Western blotting analysis confirmed that the CP of DsMV is considerably larger than those of most potyviruses (Abo El-Nil et al., 1978). The CP MWs of six DsMV isolates were estimated to be 38-47 kDa, whereas ten other potyviruses used in comparisons had estimated MWs of 31-36 kDa in Western blotting analyses. These variations apparently reflect genomic differences between DsMV isolates since the specific CP MW for each isolate is constant after serial passages through different hosts. Whereas most potyviruses, including konjak mosaic (Shimoyama et al., 1992), have CP MWs of about 32-36 kDa, those of DsMV isolates have values of 38-47 kDa. Abo El-Nil et al. (1977) suggested that the high CP MW values of DsMV was associated with the N-terminal portion of the coat protein. The availability of the CP sequences of several DsMV isolates (Pappu et al., 1994a, b; this study) and of other potyviruses may help to confirm this hypothesis. Indeed, the DsMV CPs of 314 to 330 amino acids noted in this study are

```
    1                                                          50
DsMV-Ch1  AGGTCTGGTA AACAGGGCCG ACAGTTATTG GCTCGCTGTT TGTAGTTTTA
DsMV-Ch1a AGGTCTGGTA AACAGG..CC ACAGTTATTG GCTCGCTGTT TGTAGTTTTA
DsMV-Ch2  AGGTCTGGTA AACAGG..CC ACAGTTATTG GCTCGCTGTC TGTAGTCTTA
DsMV-LA   AGGTCTGGTA AACAGA..GA CCACTTATCG TCTCGCTGTC TGTAGTTTTA

          51                                                   100
          TTTATATAAA GTATTGTTTG TATTCAAGTA GTGCTATTTG GTTATAAACT
          TTTACATAAA GTATTGTTTG TATTCAAGTA GTGCTATTTG ATTATAAACT
          TATATTTAAA GTACTGTTTG TATTCAGGTA GTGTTATTTG ATTATAAACT
          TATATTTAAA GTACTGTTTG TTTTCAAGTT GTGGTATTTG ATTACAAACT

          101                                                  150
          ACAGCGTGGT TTTCCACCGA TGTGGAGTTG .GCTTTGCAC CCTATTATCT
          ACAGAGTGGT TTTCCACCGA TGTGGAGA.G TGCTATGCAT CCTACTATCT
          ACAGTGTGTT TTTCCACCGA TGTGGAGAGG TGCTATGCAC CCTACTATCT
          ACAGTGTGTT TTTCCACCGA TGTGGAGAGG TGCTATGCAC CCTACTATCT

          151                                                  200
          AC.GTCCTTT ATGTATTTGA AAACTACTGA ACTACTGCAC CTACGTCAGA
          ACATTCCTTT AAATGTTTGA AAACTACTGA ACCACTGCAC CTACATCAGA
          AC.GTCCTTT AAATATTTGG AAACTGCTGA ACCACTGCAC CTACATCGGA
          AC.GTCCTTT AAATATTTGG AAACTGCTGA ACCACTGCAC CTACATCGGA

          201                                                  250
          CCGCAAG... GCGATGGGCG CGGTAGGCGA GACGCTTCGT GCACGGTGTT
          CCGTAAG... ..CCATGGCG CTGTAGGCGA GATGCTTCGT GCACGGTGTT
          CCGTTGGTGC GCCACTGGCG CGGTAGGCGA GATGCTTCGT GCACGGTGTT
          CCGTTGGTGC GCCACTGGCG CGGTAGGCGA GATGCTTCGT GCACGGTGTT

          251
          C
          C
          .
          .
```

Fig. 3-7. Comparison of nucleotide sequences of the 3'-NCRs of dasheen mosaic virus isolates Ch1 and Ch1a, Ch2 and LA. Dots indicate the gaps for optimum alignment.

relatively large for a potyvirus. However, the calculated CP MWs for four sequenced isolates were from 34.6-36.9 kDa, which were close to those seen for other potyviruses. Furthermore, the estimated MW of the CP expressed in *E. coli* was smaller (39 kDa, as reported in chapter 2) than that (44 kDa) from infected plants, even though the expressed CP had a fusion protein of 15 amino acids long. The similar differences between CP MW (36 kDa) observed in SDS-PAGE and that calculated using sequence data (33 kDa) have been reported for PRSV-P (Quemada et al., 1990a; Yeh et al., 1992).

There are several factors that could account for such apparent discrepancies in MW. First, they might reflect the unusual amino acid composition of the DsMV CP (Pappu et al., 1994a; Li et al., 1994; this study). The DsMV CP is different from those of other potyviruses in that it is quite threonine and asparagine-rich at the N-terminal region. These two amino acid residues account for 31.8-50% of the amino acid residues at the N-terminal region of the CPs. In addition to a 6-proline sequence at the N-terminal region, there are also many more (8-10) potential *N*-glycosylation sites which are clustered near both the N- and the C-termini of the DsMV CP (Pappu et al., 1994a; this study) than in the CPs of most potyviruses. These unusual sequences may affect behavior of DsMV CP in SDS-PAGE, although theoretically no such influence should exist based on the presumption that proteins are completely denatured under such conditions. Several short proline stretches and/or a seven-proline stretch were also found at the N-terminal regions of several sweet potato potyviruses (Colinet & Lepoivre, 1994). The CP MWs of these viruses in SDS-PAGE, however, corresponded closely to those calculated from their

sequences of 316-355 amino acids, thereby indicating that proline stretches had no effect on the MWs of the proteins in SDS-PAGE.

Another possibility is that some chemical components in aroids may have effects on the migration rate of the DsMV CP in SDS-PAGE, causing such an abnormal behavior. This possibility could be not tested, however, because the host range of DsMV is largely restricted to aroids. Clearly, attempts to purify this virus from different aroids were seriously impeded by their unusually viscous sap.

The variability of the CP MWs among different isolates, contrasts with the studies of other potyvirus strains, such as ZYMV (Wisler, 1992) and PRSV (data not shown), which appear to be much more uniform in size. Sequence analysis of the DsMV CPs revealed a deletion and an addition at the N-terminal regions of the Ch1, Ch2 and TEN isolates, which, when compared to the LA isolate, consisted of a 16-amino-acid deletion. The deletions or duplications at the N-terminal region of the DsMV CP among different isolates may contribute to the variability of the coat protein sizes. The significance of these deletions or duplications to the evolution of DsMV has been discussed by Pappu et al. (1994). Similar sequence diversities at the N-terminal region of the CPs were also reported for strains of TuMV (Sano et al., 1992), SCMV (Xiao et al., 1993), and BCMV (Khan et al., 1993).

The availability of the sequences of the coat protein and the 3'-NCR of the DsMV isolates, Ch1, Ch2, LA and TEN, allowed an assessment to be made of their relationship at the sequence level. As has been for reported for other potyviruses (Shukla et al., 1988),

the amino acid variation among the CPs of the four DsMV isolates occurs primarily at the N-terminal region, while the sequence of the core and C-terminal regions are highly conserved (Fig. 3-6). The CP sequences of these isolates showed similarities of 92% to 96%, which are, by convention, considered to be values delineated for strains of a given virus (Shukla et al., 1988). Furthermore, the similarities of the 3'-NCR sequences of the isolates Ch1, Ch2 and LA were 79-83%, indicating that these isolates are very close to each other.

We confirmed earlier studies (Abo El-Nil et al., 1977; Wisler et al., 1978) that some isolates of DsMV can induce more severe stunting symptoms in *P. selloum* than others, but CP MW is apparently not correlated with this properties. For example, the respective isolates with the highest and lowest CP MW values, DsMV-Xc (47 kDa) and DsMV-Ch3 (38 kDa) induced mild symptoms in *P. selloum*, whereas the four other isolates (Ch1, Ch2, Ce1 and Za) that induced more severe stunting symptoms had intermediate CP MWs. However, relationships between the symptom differences in *P. selloum* and CP sequence similarity of these isolates could not be established since CPs of the Ch3, Xc and Za isolates have yet to be sequenced.

The coat protein variability of DsMV isolates does not appear to compromise the ability to detect different DsMV isolates serologically. Abo El-Nil et al. (1977) noted strong precipitin reactions between isolates with only barely perceptible reciprocal spur formation in SDS-immunodiffusion tests. Likewise, each of the DsMV isolates could

readily be detected in ELISA and Western blot tests in this study, as could several taro isolates of DsMV isolates from different geographical areas (Zettler et al., 1987).

Despite the CP MW differences between DsMV and other potyviruses (except two PMoV strains) reciprocal reactions in Western blotting analyses reveal close serological relationships. The unusual composition of its CP apparently had no significant effect on the ability of DsMV antiserum to react against many other potyviruses. Furthermore, the antisera from two different DsMV isolates cross-reacted with other potyviruses, thereby providing additional evidence that the unusual amino acid residues stretches at the DsMV CP does not contain epitopes essential for detecting it serologically.

CHAPTER 4
DETECTION OF DASHEEN MOSAIC VIRUS IN AROID CROPS

Introduction

Plants infected with dasheen mosaic virus (DsMV) often express symptoms intermittently (Zettler et al., 1978; Chase & Zettler, 1982), which presents a problem for the detection of the virus based on visual symptoms alone. Also, DsMV is perpetuated and carried over long distances through symptomless vegetative propagating materials. Development of reliable and practical methods for detecting DsMV and effective therapeutic procedures are essential for the international movement of disease-free aroid plants, especially if severe isolates are involved (Jackson, 1982). Bioassays (growout tests and indicator hosts), electron microscopy (EM) and immunodiffusion tests have been the methods most commonly used previously for the detection of DsMV (Zettler & Hartman, 1986), although more recently, enzyme-linked immunosorbent assay (ELISA), immunosorbent electron microscopy (ISEM) and immuno-dot blotting for DsMV detection have also been used (Hu et al., 1995; Rodoni & Moran, 1988; Zheng et al., 1988; Ko et al., 1986; Kositratana, 1985). Bioassay methods pose practical problems for DsMV detection since this virus is largely confined to plants in the family Araceae, and suitable indicator plants are not readily available (Zettler & Hartman, 1986). Moreover, the bioassay methods are laborious, time-consuming, and require extensive greenhouse space (Matthews, 1991). Seed of *Philodendron selloum*, the most widely used test

species, is available only on a seasonal basis and this species is not a local lesion host. *Philodendron verrucosum*, a local lesion host (Tooyama, 1975), is a relatively rare plant species, and it is not commercially available.

The antigenic properties of many plant viruses make it possible to detect them with a high degree of sensitivity and specificity. A number of serological methods, such as immunodiffusion tests, ISEM, ELISA, Western blotting, and immuno-dot blotting have been developed for detection of plant viruses. Over the years, these techniques have been refined, and there is an increasing availability of polyclonal and monoclonal antisera for conducting these tests (Speigel et al., 1993).

Reverse transcription polymerase chain reaction (RT-PCR)-based assays have been increasingly used for detection of plant RNA viruses due to their high sensitivities (Vunsh et al., 1990; Wetzel et al., 1991a; Langeveld et al., 1991; Robertson et al., 1991; Rojas et al., 1993; Rowhani et al., 1993; Colinet et al., 1994; Smith et al., 1994). With its relative simplicity and high sensitivity, RT-PCR has good potential for detecting minute quantities of virus in plant tissue. Determination of the 3'-terminal region of the DsMV genome (this study) has provided us with the opportunity to detect this virus by RT-PCR.

Materials and Methods

DsMV Isolates

DsMV isolates from different hosts were collected and maintained in greenhouses for study. The caladium *(Caladium hortulanum)* corms were collected from a commerial source in

Highlands County, Florida. Cocoyam (*Xanthosoma caracu)* corms were from Costa Rica; and the taro (*Colocasia esculenta)* corms were from Taiwan. Leaves of wild *C. esculenta* plants were also collected from the campus of the University of Florida at Gainesville. The calla lily (*Zantedeschia aethiopica*) plants were from Watsonville, CA. DsMV isolates from these plants were mechanically transmitted to *Philodendron selloum* seedlings. The infected plants developed systemic chlorotic streaking along the veins of new leaves within 2-3 weeks after inoculation.

Immunosorbent Electron Microscopy (ISEM)

Immunosorbent electron microscopy (ISEM) was performed by chopping a small piece of leaf tissue into tiny pieces with a razor blade in a drop of 50 mM potassium phosphate buffer, pH 7.2. The extract was incubated on a grid for 1 min, and then rinsed with potassium phosphate buffer and water. DsMV-FL virion antiserum (Abo El-Nil et al., 1977) diluted 1:200 was added on the grid and incubated for 5 min. Following washing, protein A gold-labeled solution was applied on the grid for 5 min. After washing, the grid was stained with 2% uranyl acetate and viewed with a Hitachi H-600 transmission electron microscope. For thin sections, leaf samples (1 x 2 mm) of *P. selloum,* caladium and cocoyam plants infected with DsMV were fixed in 4% glutaraldehyde (in 0.1 M potassium phosphate buffer, pH 7.2), post-fixed with 1% osmium tetroxide, dehydrated in an acetone series, and embedded in Spurr's epoxy resin. Sections were cut with a Sorvall MT2-B ultramicrotome (Du Pont Co., Wilmington, DE) and then stained with uranyl acetate and lead citrate.

Enzyme-linked Immunosorbent Assay (ELISA)

The DsMV antisera used in the ELISA tests were the DsMV-FL antiserum used by Abo El-Nil et al. (1977) and the antiserum prepared in this study to CP expressed in *Escherichia coli*. The PTY 1 cross-reactive monoclonal antibody was also used. The anti-rabbit and antimouse IgG used was obtained by the Sigma Chemical Co. (St. Louis, MO). The ELISA procedure was as described by Clark and Adams (1977). To purify IgG from the DsMV-FL virion antiserum, one ml of antiserum was diluted in 9 ml of distilled water. The IgG was precipitated by adding an equal volume of saturated ammonium sulfate solution and incubating it at room temperature for 30-60 min. The solution was then centrifuged at 10,000 *g* for 10 min. The IgG precipitate was resuspended in 2 ml 1/2 strength phosphate-buffered saline (PBS, 20 mM sodium phosphate-potassium phosphate buffer, pH 7.4, containing 3 mM potassium chloride and 150 mM sodium chloride, pH 7.4) and dialyzed three times against 1/2 strength PBS at 4°C for 4 hr. The IgG was then fractionated through a DEAE Sephacel column (Pharmacia, Uppsala, Sweden). The effluent was collected in fractions of 1 ml and monitored by a spectrophotometer at 280 nm. The first peak of protein was collected and adjusted to 1.4 OD. Purified DsMV-FL IgG was used either immediately for enzyme conjugation or stored at -20°C for use as coating antibody. Type VII alkaline phosphatase (Sigma Chemical) was centrifuged at 1,000 *g* for 10 min and the pellet was resuspended in purified IgG preparation at a concentration of 1 mg/ml and at a ratio of 1:2 (enzyme:IgG). The mixture was then dialyzed against PBS at 4°C, and then 25% aqueous glutaraldehyde was

added to make a final concentration of 0.05%. The mixture was incubated at room temperature for 4 hr. After dialysis, bovine serum albumin (Sigma Chemical) was added to a final concentration of 5 mg/ml. The conjugate was stored at 4°C for ELISA tests.

In indirect ELISA (I-ELISA) tests, samples were ground in CEP buffer [15 mM sodium carbonate, 35 mM sodium bicarbonate, 0.02% polyvinyl pyrrolidone (PVP), and 0.2% egg albumin (EA)] or coating buffer (12 mM sodium bicarbonate, 35 mM sodium carbonate, pH 9.6) in a ratio of 1 to 10, and added to the wells of the Type II polystyrene microelisa plates (Dynatech Labs, Inc., Chantilly, VA). For each well, 100 µl of sample was added. The plates were incubated at 37°C for 1-2 hr and rinsed with four 5-minute washes in PBS, containing 0.05% Tween-20 (PBST). Fifty µl of DsMV antibody diluted 1:500 (DsMV-FL antiserum) or 1:5000 (*E. coli* expressed CP antiserum) or 1:1000 (PTY 1) in enzyme-conjugate buffer (PBST, containing 2.0% polyvinyl pyrrolidone and 0.2% ovalbumin), was added and incubated at 37°C for 1-2 hr. The plates were then rinsed four times as before. Fifty µl of 2 µg/ml antirabbit IgG (for DsMV polyclonal antisera) or antimouse IgG (for monoclonal antiserum), each diluted 1:3,000 in enzyme-conjugate buffer, was then added to appropriate wells. The plates were incubated for 1-2 hr at 37°C and washed 5 times in PBST. Fifty µl of substrate (ρ-nitrophenyl disodium phosphate, 1 mg/ml, Sigma Chemical) in substracte buffer at pH 9.8 (9.7% diethanolamine, Fisher Scientific, Fair Lawn, NJ) was added to the plates and incubated at room temperature. Absorbance readings (405 nm) were taken on a Biotek automated microplate reader, model EL 309 (Bio-Tek Instruments Inc., Winooski, VT) at fifteen minute intervals for 1 hr.

For DAS-ELISA, coating buffer (200 µl) was added to plates and incubated for 1 hr at 37°C. The plates were then washed 3 times with PBST. One hundred µl of DsMV-FL antiserum diluted 1:500 in the enzyme-conjugated buffer was added to the plates and incubated at 37°C for 1-2 hr. The plates were washed four times with PBST. Fifty µl of the prepared antigen was added to the plates and incubated at 37°C for 1-2 hr. The plates were washed four times in PBST, and fifty µl of phosphatase-conjugated DsMV IgG was then added to the plates. The plates were incubated at 37°C for 1-2 hr and washed five times in PBST. The remainding steps were as described previously.

Western Blotting

Virus was detected by Western blotting as previously described (Li et al., 1990). Leaf or petiole tissue (0.1 g) was minced and added (1:2.5, w/v) to SDS extraction buffer (62.5 mM Tris buffer, pH 6.8, containing 2% SDS, 10% glycerol, and 5% ß-mercaptoethanol) in a 1.5-ml microfuge tube. The samples were boiled for 2 min. The supernatant was saved and stored at -20°C. Extracts were run through 10% SDS-acrylamide gel on a Mini-Protean II Electrophoresis Cell (Bio-Rad Laboratories, Inc., Melville, NY) for 1.5 hr at 100-V constant voltage. Separated proteins were transferred to nitrocellulose membranes (0.45µ pore size, from Bio-Rad Laboratories) by electroblotting in a Bio-Rad Mini Trans-Blot Cell (Model 200/2.0) for 1 hr. The blots were washed three times for 5 min in TBST buffer (20 mM Tris, pH 7.5, containing 0.5 M NaCl and 0.05% Tween-20), soaked for 1 hr at room temperature in 1:500-5,000 dilution of antisera, and again washed three times, each for 5 min with TBST. The

blots were soaked at room temperature for 1 hr in a solution containing alkaline phosphatase-conjugated goat antirabbit or antimouse IgG (1:1,000 dilution). The conjugate was detected with 0.3 mg/ml nitro blue tetrazolium (Fisher Scientific, Pittsburgh, PA) and 0.15 mg/ml 5-bromo-4-choloro-3-indolyl phosphate (Fisher Scientific) in developing buffer, pH 9.6 (0.1 M $NaHCO_3$, containing 0.1 M $MgCl_2$). The reaction was stopped by rinsining in deionized water. Prestained protein standards (Gibco BRL, Gaithersburg, MD) were used as markers.

Corm Wounding Test

Twenty caladium corms were cut with a razor blade as described by Vunsh et al. for gladiolus (1990). Part (0.1 g) of the corm tissue was tested immediately by IEM and I-ELISA. The remainder of the sample was put into a paper bag, and kept at room temperature. Twenty days later, the corm tissue near the wounded surface was tested by the same methods.

Reverse Transcription-Polymerase Chain Reaction (RT-PCR)

Total RNA was isolated from leaf tissue of either infected or healthy plants according to the following method described by de Varies et al. (1988). All solutions were prepared by diethyl pyrocarbonate (DEPC)-treated water and autoclaved. Leaf tissues (0.4 g) of DsMV-infected and healthy plants were ground in liquid nitrogen into fine powder. Two volumes (0.8 ml, v/w) of RNA extracting buffer (0.1 M Tris-HCl, pH 8.0, containing 0.1 M LiCl, 10 mM EDTA and 1% SDS) and two volumes (0.8 ml, v/w) of hot phenol (70°C) were added to the frozen powder, ground briefly and transferred to a 2-ml microtube. The tube was heated at

70°C for 5 min and centrifuged for 10 min at 12,000 *g* in an Eppendorf microcentrifuge (Brinkmann Instruments, Inc., Westbury, NY). The supernatant was recovered and extracted with phenol/chloroform (1:1; v/v) three times. The phenol was removed by chloroform extraction. The total RNA was precipitated from the recovered aqueous phase by addition of 1/3 volume of 8 M LiCl at 4°C overnight. The mixture was centrifuged at 12,000 *g* for 15 min. The pellet was washed sequentially in 2 M LiCl and 70% ethanol, vacuum-dried, and resuspended in 50 µl of sterile distilled water (5 Prime to 3 Prime Inc., Boulder, CO). Five µl aliquots of total RNA were used immediately to synthesize cDNA, and the remaining aliquots were stored at -80°C for future use.

Reverse transcription was performed on 10 µg of total RNA extracted from leaves in a reaction volume of 20 µl containing 1x reaction buffer (50 mM Tris-HCl, pH 8.3, 75 mM KCl, 3 mM $MgCl_2$), 10 mM dithiothreitol (DTT), 100 µM each of four deoxyribonucleotide triphosphate (dNTPs), 20 units RNasin (Promega Co., Madison, WI), 100 units Moloney murine leukemia virus reverse transcriptase (SuperScript II, from Gibco BRL), and 100 pmol downstream primer EH258-260 (5'-TTTTTTTTTTTTTTTTTTTTA/C/G-3'). This mixture was incubated for 60 min at 41°C. The reaction was terminated by incubating mixture at 95°C for 10 min. and then cooling it on ice. The synthesized cDNA was used immediately as the template for PCR amplification.

The PCR was carried out in a UNO-Thermocycler (Biometra Inc., Tampa, FL). The primers, EH232 (5'-AAGCTTGCAGGCTGATGATACAG-3') corresponding to the 5'-end of the DsMV CP gene and linked at the 5'-end of it a *Hind* III restriction site, and EH234 (5'-

GAATTCTTGAACACCGTGCAC-3') corresponding to the 3'-end of the non-coding region and linked at the 5'-end of it a *Eco*RI restriction site, were used for amplification of the gene. For standard reaction, 200 μM dNTPs, 0.2 μM of each primer, 1x PCR buffer (10 mM Tris-Hcl, pH 8.2, containing 50 mM KCl), 1.5 mM $MgCl_2$, 2.5 U of *Taq* DNA polymerase, 200 pmol of each primer, and 2 μl of cDNA products were used in a total of 100 μl reaction volume. The reactions were run at the following temperature-cycling profile (touchdown PCR): 11 cycles of 1.5 min at 94°C, 2 min at 72→61°C (temperature declines of 1°C each cycle), and 2 min at 72°C; 24 cycles of 1 min at 94°C, 1 min at 61°C, and 2 min at 72°C followed by a 10 min extension at 72°C.

The following temperature-cycling profile (regular PCR) was applied in the primary reactions or in subcloning of DsMV CP using plasmid DNA as template: 5 cycles of 1.5 min at 94°C, 2 min at 61°C, 2 min at 72°C; 30 cycles of 1 min at 94°C, 1 min at 61°C, and 2 min at 72°C, followed by a 10 min extension at 72°C. Ten μl of the PCR products were then run on a 0.9% agarose (Gibco BRL) electrophoresis gel. Gels were stained for 5 min with 0.5 μg/ml ethidium bromide, destained in deionized water for 15 min, and photographed on a UV-transilluminator with a Gel Print ToolBox (BioPhotonics Co., Ann Arbor, MI).

Southern Blotting

Nucleic acid probes prepared from DsMV CP fragments amplified from plasmid pCP1 by PCR were labeled with (^{32}P) dCTP (Du Pont NEN, Boston, MA) using a Random Primed DNA KIT (Boehringer Mannheim, Indianapolis, IN) according to the manufacturer's

instructions. The RT-PCR products were analyzed on a 0.9% agarose gel. The gel was soaked in 50 ml of solution (1.5 M NaCl, 0.625 N NaOH) for 8 min, then in 50 ml of a neutralized solution (1 M Tris-HCl, pH 7.4, 1.5 M NaCl), and equilibrated with 10x standard saline citrate (SSC) (1.5 M NaCl, 0.15 M sodium citrate, pH 7.0). The RT-PCR products were then transferred to a Nylon Hybond membrane with a 0.45 μ pore size (Amersham, Inc., Arlington Heights, IL) by blotting in 10x SSC for 3 hr. Nucleic acids were bound to the membrane by UV cross-linking using a UV-Crosslinker FB-UVXL-1000 (Fisher Scientific) for 90 sec. The membrane was prehybridized in a rotating Mini Oven (Vangard International, Inc., Neptune, NJ) at 65°C for one hour with 20 ml of 5x SSC, containing 1% SDS, 10% PEG 8000 and 1 mg/ml denatured salmon sperm DNA. Approximately 100-200 μCi/ml denatured probe was added to the prehybridization solution and allowed to hybridize to the membrane overnight at 65°C. After hybridization, the solution was stored at -20°C in a sterile tube and reused within one month. Before reuse, the probe was denatured by boiling for 5-10 min. The membrane was washed twice in 100 ml of 2x SSC for 5 min each at room temperature, followed by two rinses in 100 ml of 0.4x SSC containing 2% SDS at 65°C for 30 min each. A final rinse was in 100 ml of 0.2x SSC at room temperature for 30 min. Washed membranes were exposed to X-ray film with an intensifying screen.

Comparison of I-ELISA, Western Blot and RT-PCR

Leaf tissues from DsMV infected calla lily and healthy *P. selloum* seedling plants were used to compare the sensitivities of I-ELISA, Western blotting and RT-PCR. For I-ELISA and

western blotting, leaf extracts diluted 1:10 with buffer (equal to 10 mg tissue) were used. Ten µg of total plant RNA (extract of 10 mg tissue) were used for RT-PCR. The methods for these tests were as those described above for each of the respective techniques.

Results

Cylindrical Inclusions of DsMV

Type III cylindrical inclusion bodies with scrolls, pinwheels and laminated aggregates as described previously for DsMV (Zettler et al., 1978) were seen in cells of 'Candidum' caladium and cocoyam infected with, respectively, the Ch1 and Xc isolates of DsMV (Fig. 4-1).

ELISA

In direct comparisons, I-ELISA was found to be more sensitive than DAS-ELISA (Table 4-1). The OD values of I-ELISA were as much as four times those of DAS-ELISA. Although the background reaction was lower when 0.5 µg/ml DsMV IgG was used, use of 2 µg/ml IgG resulted in significant contrast between infected and healthy samples within a 15 min observation time. Similar results were noted in I-ELISA tests regardless whether extraction was in coating buffer or CEP buffer was used. Accordingly, I-ELISA using CEP as the extracting buffer was routinely used to detect DsMV in tissue culture-derived plantlets.

The sensitivity of I-ELISA was very similar to that of Western blotting when both methods were compared (Table 4-2). Eight out of 10 'Candidum', 5 of 14 'Frieda Hemple'

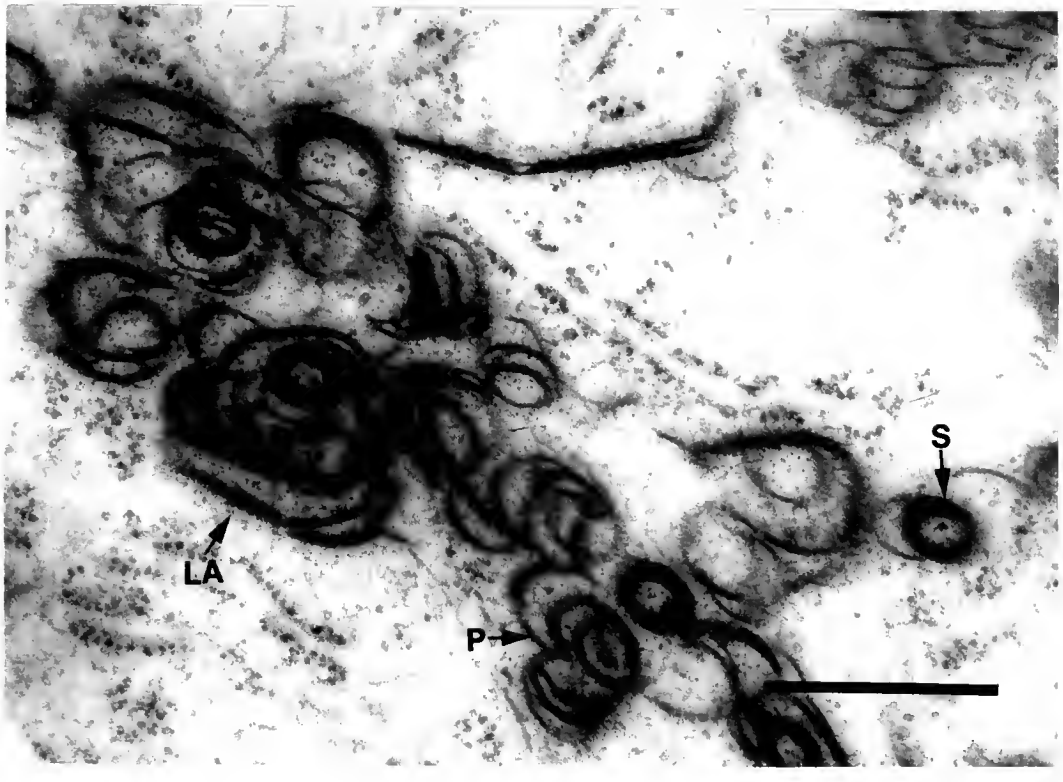

Fig. 4-1. Electron micrograph of cylindrical inclusions (CI) induced by a caladium isolate of dasheen mosaic virus in a leaf cell of *Philodendron selloum*. Typical scrolls, pinwheels, and laminated aggregates were presented. Many virus particles were in the cytoplasm of the infected cell. LA, laminated aggregate; P, pinwheel; S, scroll. Bar = 1 μm.

Table 4-1. A_{405} absorbance values of I-ELISA[1] and DAS-ELISA[1] for DsMV detection

Test	Sample[2]							
	1	2	3	4	5	6	7	8
I-ELISA(CEP[3])	0.492	0.492	0.458	0.544	0.612	0.001	0.014	0.009
I-ELISA (CB[3])	0.356	0.102	0.490	0.225	0.482	0.017	0.012	0.009
DAS-ELISA	0.153	0.148	0.315	0.056	0.153	0.003	0.006	0.000

[1] I-ELISA = indirect ELISA and DAS-ELISA = double antibody sandwich direct ELISA.

[2] Antigens used were extracts from leaf samples of caladium plants grown out from commercial corms. Antigens 1:10 in buffer and DsMV-FL antiserum 1:500 in buffer. Absorbance readings were taken 15 min after adding substrate. The absorbance (A_{405}) value represents the mean of six wells.

[3] CEP = coating extraction phosphate buffer and CB = coating buffer.

Table 4-2. Comparison of I-ELISA[1] and Western blotting[2] procedures to detect DsMV in caladium leaves

Cultivar	No. of samples	I-ELISA	Western blotting
Candidum	8	+	+
Candidum	2	-	-
Frieda Hemple	5	+	+
Frieda Hemple	9	-	-
Carolyn Whorton	1	+	+
Carolyn Whorton	10	-	-
Control[3]	3	-	-

[1] I-ELISA was performed as described in the text. Leaf tissues of plants were ground 1:10 in CEP buffer and DsMV-FL antiserum was diluted 1:500 in enzyme-conjugate buffer. The absorbance (A_{405}) value for positive sample was at least three times higher than that of healthy control.

[2] Western blotting was carried out as described in the text. Leaf tissues of plants were used. DsMV-FL antiserum diluted 1:500 in TBST buffer was used to detect DsMV. A protein band of the CP size of each isolate was detected in positive sample in western blotting analysis, while no such band was detected in the negative samples or healthy controls.

[3] Plants used as controls were tissue culture-derived caladium plants.

and 1 of 15 'Carolyn Whorton' caladium plants were determined to be infected by both methods.

Distribution of Detectable DsMV within Infected Plants by I-ELISA

Leaves, petioles, and/or corms of three naturally infected hosts (caladium, taro and cocoyam) were collected and tested by I-ELISA using DsMV-FL antiserum. DsMV was detected reliably in both leaves and petioles of infected caladium plants, while in cocoyam and taro plants, DsMV sometimes was detected in leaves, but not in petioles or vice versa (Table 4-3). Although DsMV usually was detected in both leaves and petioles of infected caladium plants, it was not always detected in corms of the same plants.

DsMV was detected in leaves of infected caladium plants whether symptoms were evident or lacking. In contrast, only symptomatic tissues of infected cocoyam and taro plants reacted positively in I-ELISA. Whereas symptomatic leaves of greenhouse-grown plants were evident throughout the 3-year observation period, those of cocoyam and taro were evident only in the early spring or late fall months of each year.

Corm Wounding

Corm wounding facilitated the ability to detect DsMV in caladium. Similar results were obtained by Vunsh et al. (1990) for BYMV in gladiolus. Whereas DsMV was detected by I-ELISA and ISEM in only 1 of 20 unwounded corms, it was detected in 13 of the same corms 20 days after wounding (Table 4-4).

Table 4-3. Relative distribution of DsMV in three aroid hosts[1] as determined by I-ELISA

Host	No. Samples	Leaf	Petiole	Corm
Caladium	22	+	+	+
	5	+	-	-
	1	-	-	-
Cocoyam	12	+	+	nt[2]
	2	+	-	nt
	2	-	+	nt
	17	-	-	nt
Taro	1	+	+	nt
	2	+	-	nt
	1	-	+	nt
	2	-	-	nt
Control[3]	3	-	-	-

[1] Corm tissues were tested before planting in pots. The leaf and petiole tissues were collected from plants from the tested corms. DsMV-FL antiserum tested by I-ELISA as described in the text was used. The absorbance (A_{405}) value for positive samples were at least three times higher than that of the controls.

[2] nt = not tested.

[3] Plants used as controls were tissue culture-derived caladium plants.

Table 4-4. Effect of wounding on detection of DsMV in caladium corms[1]

No. of Corms[2]	Unwounded Corm		Wounded Corm	
	I-ELISA[3]	ISEM[3]	I-ELISA	ISEM
1	+	+	+	+
7	+	-	+	+
1	-	+	+	+
4	-	-	+	+
7	-	-	-	-

[1] Corms of `Frieda Hemple' caladium were used in tests. Corms were cut and kept in a paper bag at room temperature. The corm tissues were tested immediately before wounding and 20 days after wounding.

[2] represents the number of corms which gave the indicated reaction in both I-ELISA and ISEM tests. For I-ELISA tests, the absorbance (A_{405} nm)of the positive sample was at least three times higher than that of the healthy control. Viral particles were detected in positive samples, but not in negative samples by electron microscopy.

[3] DsMV was detected by I-ELISA and ISEM using DsMV-FL antiserum.

RT-PCR for DsMV Detection

A DNA fragment of about 1200 bp, which represented the CP gene and the 3' non-coding region, was resolved by agarose gel electrophoresis from infected caladium plants. No comparable product was amplified from healthy *P. selloum* seedlings (Fig. 4-2). Strong amplification of a second smaller DNA fragment of about 500 bp was observed when reactions were run at the regular temperature-cycling profile (61°C as annealing temperature) (Fig. 4-2A). However, the second non-specific band disappeared when the "touchdown" temperature-cycling profile (72→61°C as annealing temperature) was assessed (Fig. 4-2B).

Comparison between RT-PCR, ELISA, and Western blotting for DsMV Detection

Infected caladium and field-collected calla lily plants were used to compare the sensitivity of RT-PCR for DsMV detection. Healthy *P. selloum* seedlings and pDCP1 plasmid were used as negative and positive controls, respectively. An amplified fragment of the expected size was obtained from all infected caladium and calla lily samples, whereas no fragment was amplified from the negative controls (Fig. 4-3A). The results of RT-PCR were confirmed by southern blotting using a radioactive probe (Fig. 4-3B). However, DsMV was not detected in 3 of the 8 infected calla lily samples when I-ELISA and Western blotting tests were used (Table 4-5).

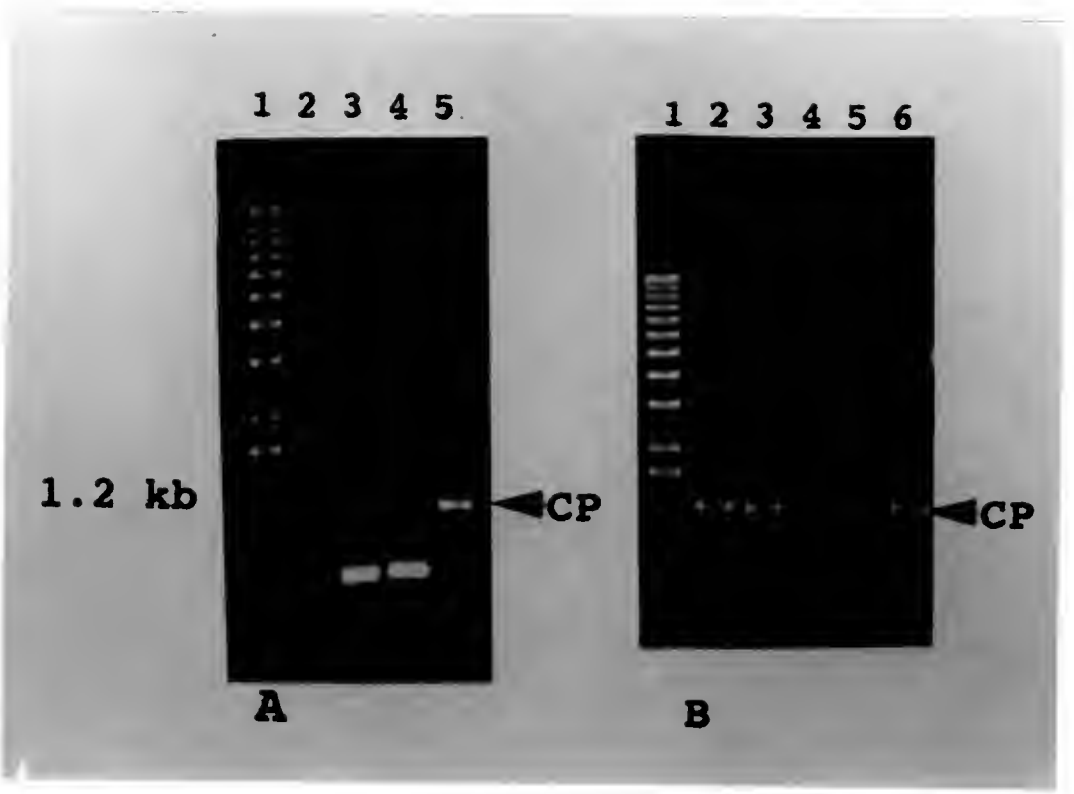

Fig. 4-2. Agarose gel electrophoresis of RT-PCR amplified products obtained from total RNA extracted from aroid leaf tissues. A. Lane 1, 1 kb DNA ladder; lane 2, healthy caladium; lanes 3-4, DsMV-infected caladium; lane 5, pDCP1 plasmid. The oligonucleotide primer used for cDNA synthesis was EH258-260, and for PCR the DsMV CP primers EH232 and EH234 were used. The amplified fragment of 1200 bp contained the intact DsMV CP gene. The CP was detected using the following temperature-cycling profile in a UNO-Thermocycler (Biometra Inc., Tampa, FL): five cycles of 1.5 min at 94°C, 2 min at 61°C, 2 min at 72°C; 30 cycles of 1 min at 94°C, 1 min at 61°C, 2 min at 72°C. B. Lane 1; 1 kb DNA ladder; lanes 2-3, infected cocoyam; lane 4, infected caladium; lane 5, healthy caladium; lane 6, pDCP1 plasmid. The temperature cycling profile used: eleven cycles of 1.5 min at 94°C, 2 min at 72→61°C (temperature declines 1°C each cycle), 2 min at 72°C; 24 cycles of 1 min at 94°C, 1 min at 61°C, 2 min at 72°C.

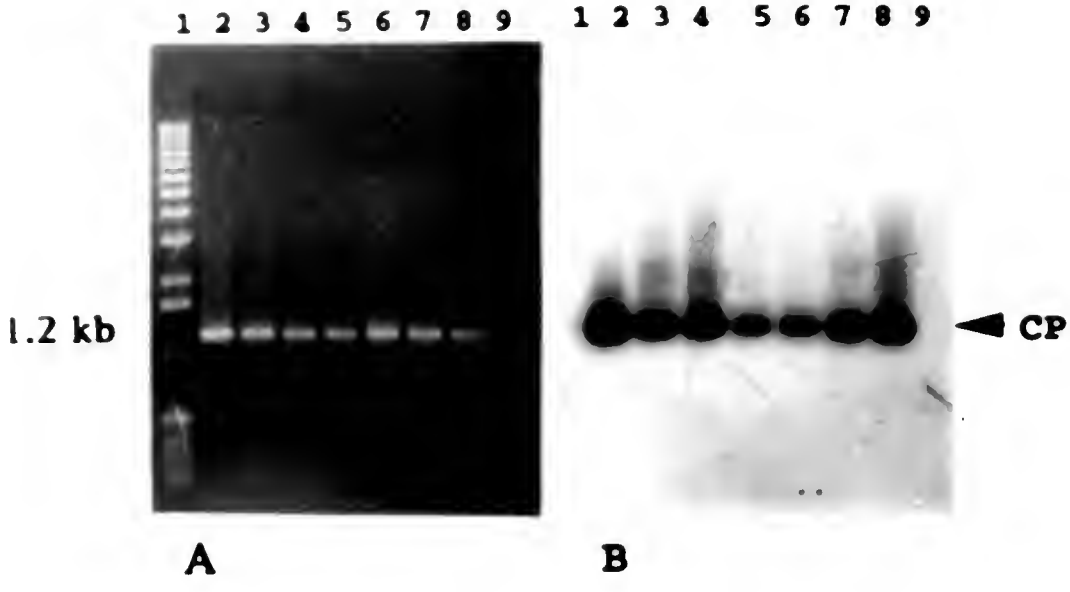

Fig. 4-3. Agarose gel electrophoresis of RT-PCR amplified products obtained from total RNA extracted from leaf tissues of calla lily plants. A. Lane 1, 1 kb DNA ladder; lane 2, DsMV-infected caladium; lanes 3-7, calla lily plants; lane 8, pDCP1 plasmid, lane 9, healthy caladium. The oligonucleotide primer used for cDNA synthesis was EH258-260, and for PCR were the DsMV CP primers EH232 and EH234. The amplified fragment of 1200 bp contained intact the DsMV CP gene. The CP was detected using the following temperature-cycling profile in a UNO-Thermocycler (Biometra Inc., Tampa, FL): 11 cycles of 1.5 min at 94°C, 2 min at 72→61°C (temperature declines 1°C each cycle), 2 min at 72°C; 24 cycles of 1 min at 94°C, 1 min at 61°C, 2 min at 72°C. B. Southern blotting analysis of RT-PCR amplified products. The DNA fragments were transferred to a nylon membrane and probed with the cloned CP gene labeled with α-^{32}P.

Table 4-5. Comparison of RT-PCR, I-ELISA and Western blotting for detecting DsMV[1]

Sample[2]	ELISA[3]	Western blotting[3]	RT-PCR[3]
1	0.058	-	+
2	0.059	-	+
3	1.850	+	+
4	2.120	+	+
5	0.470	+	+
6	0.051	-	+
7	0.283	+	+
8	1.239	+	+
Positive control[4]	1.007	+	+
Healthy[4]	0.027	-	-

[1] All samples were tested by RT-PCR, I-ELISA and western blotting tests as described in text. The I-ELISA absorbance (A_{405} nm) value represents averages of 8 wells. DsMV-FL antiserum and PTY 1 monoclonal antiserum were both used in western blotting analysis, and produced similar results.

[2] Calla lily samples were collected from the greenhouse or field.

[3] For I-ELISA tests, the absorbance (A_{405} nm) of the positive sample was at least three times higher than that of healthy control. A protein band of 44 kDa was detected for a positive sample in western blotting analysis, while no such band was detected in the negative sample or healthy control. A DNA fragment of 1.2 kb representing the CP gene and the 3' non-coding region was detected in all samples except healthy control.

[4] Positive control was DsMV-infected caladium, and the heathy control was tissue culture-derived caladium.

Discussion

The goal of this research was to refine methods for improving the detection of DsMV in aroid plants. Reliable detection systems consisting of serological assays such as ELISA, Western blotting and tissue-blotting, and of nucleic acid assays such as nucleic acid hybridization and RT-PCR (or PCR for DNA viruses) have been developed for many viruses better characterized than DsMV. In particular, limited knowledge of DsMV at the molecular level and the limited availability of polyclonal antiserum for this virus have been constraints of diagnosis and detection of the virus.

Results obtained by I-ELISA for detecting DsMV were supported by Western blotting (Table 4-2). Virus infections of aroids can be detected readily by I-ELISA (Table 4-3). Thus, I-ELISA offers a reliable alternative to previously used techniques such as SDS-immunodiffusion and ISEM for detecting DsMV from leaf, petiole and corm tissues (Zettler & Hartman, 1986).

It has been reported that the natural viscosity of the aroid leaf extracts and the presence of some "unusual" substances probably caused the high level of non-specific interference in ELISA tests (Rodoni & Moran, 1988). However, there were no such effects in our I-ELISA tests when either IgG of antiserum to DsMV-FL or antiserum to expressed CP was used. Neither extracts from healthy leaves nor from healthy corm tissues produced inordinately high background values in the tests. The contamination of the DsMV immunogen with plant proteins could have accounted for the high background in the ELISA tests of Rodani and Moran's (1988) antiserum.

Based upon ELISA results, which confirmed observations of symptoms, no virus was present in symptomless tissues of cocoyam and taro, as was reported previously for dieffenbachia (Chase & Zettler, 1982). In contrast, virus distribution is much more uniform in caladium leaves. Thus, the greenhouse grow-out recommended by the FAO/IBPGR for edible aroids, does not necessarily apply to this aroid.

The differences in symptom expression and distribution of the virus in infected taro and cocoyam plants may be attributed to the restriction of virus movement within plants of cocoyam and taro. Our results also showed that DsMV was always detected in the corm tissues from the infected caladium plants (Table 4-3). Unlike with leaf tissues, the distribution of detectable DsMV in corms was uneven, which may be related to low levels of viral replication in the corms. By wounding the corms, however, the detection rate was increased greatly, probably due to an increase in viral replication in response to wounding. Thus, as with gladiolus viruses (Vunsh et al., 1990), corm wounding could facilitate the detection of DsMV.

The detection of DsMV in association with certification and clean-stock programs for edible aroids could be greatly improved by using techniques such as RT-PCR. Our results indicate that ELISA and Western blotting could not detect DsMV in symtomless leaves of cocoyam and taro, and thus RT-PCR could be adopted to complement or substitute for ELISA. Indeed, direct comparison of RT-PCR, I-ELISA and Western blotting tests demonstrated that RT-PCR amplification of viral-specific RNA was more sensitive than I-ELISA and Western blotting to detect DsMV (Table 4-5). The sensitivity of the DsMV RT-

PCR method used in this study makes it as an attractive for virus detection, especially in those hosts in which distribution of DsMV within plants varies seasonally.

CHAPTER 5
SUMMARY AND CONCLUSIONS

A caladium isolate of dasheen mosaic virus (DsMV-Ch1) was purified from inoculated *Philodendron selloum* seedlings following a protocol used for ZYMV (Wisler, 1992). The propagative host, *P. selloum,* was the only aroid host from which the virus was successfully purified. Other aroid hosts (caladium, calla lily, cocoyam, and taro) had highly viscous leaf extracts, which may cause the virus particles to precipitate irreversibly. Symptomatic leaves of all aroid hosts, however, contained high concentrations of the virus based on examination of negatively stained leaf extracts.

Clones representing most of the genomic RNA of DsMV-Ch1 were obtained by immunoscreening of a cDNA library and direct sequence mapping. The sequence of the 3'-terminal region of 3158 nucleotides was determined. Cleavage sites were located at the Q/G (amino acids 140-141) and the Q/A (amino acids 656-657) sites of the 3'-terminal region of DsMV-Ch1. The conserved cleavage sequence VXLQ/(G, orA) was found in both sites, which is similar to those of most sequenced potyviruses, especially those in the BCMV subgroup (Shukla et al., 1994).

This sequenced region contained a portion of the NIa, the NIb, the coat protein (CP) genes and the 3' non-coding region. The partial NIa protein (140 amino acids) contained the consensus sequence GXCG, which has been found in all other sequenced potyviruses (Shukla et al., 1994). This sequence is proposed to be the active site for the

protease activity of the NIa protein. The DsMV-Ch1 NIb protein, consisting of 516 amino acids, showed 72% to 85% similarity with those of other potyviruses. The consensus motifs YCHADGS and SGQPSTVVDNT-30aa-GDD, responsible for the putative RNA polymerase function of potyviruses (Kamer & Argos, 1984; Allison et al., 1986; Domier et al.; 1986; Gunasinghe et al.; 1994), were also present in DsMV.

The size of the CP of the DsMV-Ch1 isolate was 313 amino acids with a calculated MW of 34.6 kDa; this is similar to the CPs of other potyviruses (Shukla et al.; 1994). This value was somewhat smaller than expected, however, based on SDS-PAGE results (Li et al., 1992). The DsMV-Ch1 CP shared 68-82% similarity with those of other potyviruses, and has shown a close relationship with those in the BCMV subgroup. The core regions of the CPs of DsMV-Ch1 and other potyviruses are very conserved, whereas the N-terminal regions are variable. Nevertheless, the alignments of the N-terminal regions revealed proline-rich sequences as in other DsMV isolates (Pappu et al., 1994a; this study) and other potyviruses, including johnsongrass mosaic, ornithogalum mosaic, sweet potato feathery mottle, PPV and maize dwarf mosaic (Shukla et al.; 1994). The functions of these sequences are unknown. The DAG aphid-transmission triplet was also present in the N-terminal region of DsMV starting at +5 amino acid from the cleavage site. These data show that DsMV is a typical, albeit distinct member of the genus *Potyvirus* in the family *Potyviridae*.

The DsMV-Ch1 CP gene was cloned by PCR, and subcloned into an expression vector pETh-3 to generate pETh-3-CP. The expression of pETh-3-CP in *Escherichia coli*

cells produced large quantities of insoluble DsMV CP. The expressed CP was purified from cell lysates and was used as an immunogen to produce antiserum against the DsMV CP. Serological tests such as ELISA and Western blotting indicated that the antiserum produced was similar to antiserum prepared to purified virions (Abo El-Nil et al., 1977). *In vitro* expression of the DsMV CP resolved the problems of purifying this virus and thus provided an alternative way for obtaining antiserum needed for diagnostic work.

Western blotting analysis confirmed that the CP of DsMV is considerably larger than those of most potyviruses (Abo El-Nil et al., 1978). The CP MWs of six DsMV isolates were about 38-47 kDa, whereas ten other potyviruses used in comparisons had MWs of only 31-36 kDa. These variations apparently reflect genomic differences between DsMV isolates since the specific CP MW for each isolate is constant even after serial passages through different hosts. Whereas most potyviruses, including konjak mosaic (Shimoyama et al., 1992a, b), have CP MWs of about 32-36 kDa, those of DsMV isolates have values of 38-47 kDa. Abo El-Nil et al. (1977) suggested that the high CP MW values of DsMV were associated with the labile portion of the coat protein. The availability of the CP sequences of several DsMV isolates (Pappu et al., 1994b; this study) and of other potyviruses helped to confirm this hypothesis. Indeed, the DsMV CPs of 314 to 330 amino acids of two caladium isolates noted in this study are relatively large for a potyvirus. However, the calculated CP MWs for four sequenced isolates were from 34.6-36.9 kDa, which are typical of potyviruses. Furthermore, the MW of the CP expressed in *E. coli* was smaller (39 kDa) than that (44 kDa) from infected plants, even though the

expressed CP had a fusion protein of 15 amino acids long. The similar CP MW difference between SDS-PAGE and sequence data result has been reported for PRSV-P (Quemada et al., 1990a).

One of the factors that could account for such perceived differences in molecular weights in SDS-PAGE is the amino acid composition of the DsMV CP. The DsMV CP is different from those of most potyviruses in that it is quite threonine/asparagine-rich at the N-terminal region. These two amino acid residues account for 31.8-50.0% of the amino acid residues at the N-terminal region of the CPs. In addition to a 6-proline sequence at the N-terminal region, there are also many more (8-10) potential *N*-glycosylation sites clustered near both the N- and the C-termini of the DsMV CP (Pappu et al., 1994a; this study) than there are in the CPs of most potyviruses. These unusual sequences may affect behavior of DsMV CP in SDS-PAGE, although theoretically no such influence should exist based on the presumption that proteins are completely degraded by the anionic detergent, SDS. Several short proline stretches and/or a seven-proline stretch were also found at the N-terminal regions of several sweet potato potyviruses (Colinet & Lepoivre, 1994). The CP MWs of these viruses in SDS-PAGE, however, correspond closely to those calculated from their sequences of 316-355 amino acids, thereby indicating that proline stretches may have no effect on the estimated MWs of the proteins in SDS-PAGE.

The variability of CP MWs among different DsMV isolates noted in this study contrasts with the studies of other potyviruses, such as ZYMV (Wisler, 1992) and PRSV-W (data not shown), which appear to be much more uniform among different strains.

Sequence analysis of the DsMV CPs revealed a deletion and an addition at the N-terminal regions of the Ch1, Ch2 and TEN isolates, which, when compared to the LA isolate. The deletions or duplications at the N-terminal region of the DsMV CP among different isolates may also contribute to the variability of the coat protein sizes. Similar sequence diversity at the N-terminal region of the CPs was also reported for strains of TuMV (Sano et al., 1992), strains of SCMV (Xiao et al., 1993), and BCMV (Khan et al., 1993).

The availability of the sequences of the coat protein and the 3'-NCR of the DsMV Ch1, Ch2, LA and TEN isolates allowed an assessment to stufy their relationship at the sequence level. As has been reported for other potyviruses (Shukla et al., 1988), the amino acid variation among the CPs of the four DsMV isolates occurs primarily at the N-terminal region, whereas the sequences of the core and C-terminal regions are highly conserved (Fig. 4-6). The CP sequences of these isolates showed similarities of 92 to 96%, which are, by convention, considered to be within values for delineating strains of a given virus (Shukla et al., 1988). Furthermore, the similarities of the 3'-NCR sequences of the isolates Ch1, Ch2 and LA were 79-83%, indicating that these isolates are very close to each other.

The results obtained in this research confirmed earlier studies (Abo El-Nil et al., 1977; Wisler et al., 1978) that some isolates of DsMV can induce more severe stunting symptoms in *P. selloum* than others; however, the CP MW is apparently not correlated with this property. For example, the respective isolates with the highest and lowest CP sizes, i.e. DsMV-Xc (47 kDa) and DsMV-Ch3 (38 kDa) induced mild symptoms in *P. selloum*, whereas the four other isolates (Ch1, Ch2, Ce and Za) that induced more severe

stunting symptoms had intermediate CP MWs. However, relationships between the symptom differences in *P. selloum* and the CP sequence similarity of these isolates could not be established since CPs of the Ch3, Xc and Za isolates have yet to be sequenced.

The coat protein variability of DsMV isolates does not appear to compromise the ability to detect different DsMV isolates serologically. In this study, each of the DsMV isolates could readily be detected either by ELISA or by Western blotting.

Despite the CP MW differences between DsMV and most other potyviruses, reciprocal reactions in Western blot analyses reveal close serological relationships between DsMV and other potyviruses, thereby suggesting that unusual composition of DsMV CP apparently had no significant effect on its serological affinities to other potyviruses. Furthermore, the antisera from two different DsMV isolates cross-reacted with other potyviruses, thereby providing additional evidence that the unusual DsMV CP stretches do not contain epitopes that interfere with the ability to detect different strains of the virus.

Results obtained by I-ELISA in the detection of DsMV were supported by Western blotting and ISEM. Virus infections of aroids can be detected by I-ELISA. Thus, I-ELISA offers a reliable alternative to previously used techniques such as SDS-immunodiffusion and immunosorbent electron microscopy for detecting DsMV from leaf, petiole and corm tissues. It has been reported that the natural viscosity of the aroid sap combined with the presence of some "unusual" substances probably caused the high level of non-specific interference in ELISA tests (Rodoni & Moran, 1988). However, there were no such effects in our ELISA tests, regardless whether DsMV-FL antiserum or expressed CP antiserum was used.

Our results confirmed earlier findings (Zettler et al., 1986) that DsMV symptom expression is intermittent in some aroid hosts. For cocoyam and taro, the expression of the viral symptoms usually occurred in early spring or late fall months. The intermittent symptom expression and distribution of the virus in infected cocoyam and taro plants may be attributed to the restriction of the viral movement, as was previously reported for dieffenbachia (Chase & Zettler, 1982). However, in caladium, viral symptoms could be observed throughout the season. These observations were in agreement with ELISA and Western blotting studies; DsMV was not detected by these methods in symptomless leaves of cocoyam and taro, but the virus was readily detected throughout the growing season in leaves of caladium. Thus, the one crop cycle greenhouse grow-out recommended by the FAO/IBPGR for edible aroids, does not necessarily apply to caladium. By wounding the corms, the detection rate was increased greatly, probably due to the increase of viral replication. Therefore, corm wounding could facilitate the detection of DsMV in corm tissue as that reported by Vunsh et al. (1990) for gladiolus.

DsMV detection associated with certification and clean-stock programs for edible aroids could be greatly improved by techniques such as RT-PCR which can be even more sensitive than either ELISA or Western blotting. Our results showed that DsMV could be detected by RT-PCR in calla lily or cocoyam tissues which were negative for DsMV by I-ELISA or Western blotting. The sensitivity of the DsMV RT-PCR method used in this study makes it an attractive alternative for virus detection, especially for those hosts in which distribution of DsMV within plants varies seasonally, such as cocoyam and taro.

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BIOGRAPHICAL SKETCH

Ruhui Li was born in Kunming, P. R. China, on March 17, 1959. She graduated from Yunnan Agricultural University in 1982 with a B.S. in plant protection. She started work at Yunnan Agricultural Academy of Sciences as a research assistant and then was a research associate in the field of plant pathology. In 1988, she came to the Department of Plant Pathology at the University of Florida as a visiting scholar and worked under the supervision of Dr. C. L. Niblett, after which she worked under Dr. F. W. Zettler. She started a graduate program in 1990 under the direction of F. W. Zettler, working on the molecular biology and detecting techniques of dasheen mosaic virus. She expects to receive her Ph.D. degree in August of 1995. Ruhui is a member of the American Phytopathological Society.

I certify that I have read this study and that in my opinion it conforms to acceptable standards of scholarly presentation and is fully adequate, in scope and quality, as a dissertation for the degree of Doctor of Philosophy.

F. W. Zettler, Chairman
Professor of Plant Pathology

I certify that I have read this study and that in my opinion it conforms to acceptable standards of scholarly presentation and is fully adequate, in scope and quality, as a dissertation for the degree of Doctor of Philosophy.

Ernest Hiebert, Cochairman
Professor of Plant Pathology

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Dan E. Purcifull
Professor of Plant Pathology

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This dissertation was submitted to the Graduate Faculty of the College of Agriculture and to the Graduate School and was accepted as partial fulfillment of the requirements for the degree of Doctor of Philosophy.

August, 1995

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